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(54) Title: SUBSTRATES AND ASSAYS FOR β -SECRETASE ACTIVITY

(57) Abstract: The present invention is directed to novel substrates for Hu-Asp. More particularly, the invention provides peptide substrates and fusion polypeptide substrates comprising a β -secretase cleavage site. Methods and compositions for making and using the peptides are disclosed.

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SUBSTRATES AND ASSAYS FOR β -SECRETASE ACTIVITY

Field of the Invention

The present invention relates to methods and compositions for
5 identification of modulators of β -secretase activity. More particularly, the present
invention provides novel substrates for monitoring the β -secretase activity of human
Asp2 protease and methods of using the same. Such methods and compositions will
be useful in the identification of agents that modulate β -secretase activity and thus
may be used in the therapeutic intervention of disorders characterized by the
10 presence of amyloid plaques.

Background of the Invention

Alzheimer's disease (AD) causes progressive dementia with
consequent formation of amyloid plaques, neurofibrillary tangles, gliosis and
15 neuronal loss. The disease occurs in both genetic and sporadic forms whose clinical
course and pathological features are quite similar. Three genes have been
discovered to date which, when mutated, cause an autosomal dominant form of
Alzheimer's disease. These encode the amyloid protein precursor (APP) and two
proteins, presenilin-1 (PS1) and presenilin-2 (PS2), which are structurally and
20 functionally related. Mutations in any of the three proteins have been observed to
enhance proteolytic processing of APP via an intracellular pathway that produces
amyloid beta peptide ($A\beta$ peptide, sometimes referred to as Abeta), a 40-42 amino
acid peptide that is the primary component of amyloid plaque in AD (Younkin,
*Brain Pathol.*1(4):253-62, 1991; Haass, *J. Neurosci.*11(12):3783-93, 1991).

25 Dysregulation of intracellular pathways for proteolytic processing may be
central to the pathophysiology of AD. In the case of plaque formation, mutations in
APP, PS1 or PS2 consistently alter the proteolytic processing of APP so as to
enhance formation of $A\beta$ 1-42, a form of the $A\beta$ peptide which seems to be
particularly amyloidogenic, and thus very important in AD. APP localizes to the
30 secretory membrane structure including the cell surface, and has a single C-terminal

transmembrane domain. Examples of specific isotypes of APP which are currently known to exist in humans include the 695-amino acid polypeptide described by Kang *et al.* (1987), *Nature* 325: 733-736 which is designated as the "normal" APP; the 751 amino acid polypeptide described by Ponte *et al.* (1988), *Nature* 331: 525-527 (1988) and Tanzi *et al.* (1988), *Nature* 331: 528-530; and the 770 amino acid polypeptide described by Kitaguchi *et al.*, *Nature* 331: 530-532 (1988).

The A β peptide is derived from a region of APP adjacent to and containing a portion of the transmembrane domain. Normally, processing of APP at the α -secretase site cleaves the midregion of the A β sequence adjacent to the membrane and releases the soluble, extracellular domain of APP from the cell surface. This α -secretase APP processing creates soluble APP- α , which is not thought to contribute to AD. However, pathological processing of APP at the β - and γ -secretase sites, which are located N-terminal and C-terminal to the α -secretase site, releases the A β peptide. Processing at the β - and γ -secretase sites can occur in both the endoplasmic reticulum (in neurons) and in the endosomal/lysosomal pathway after re-internalization of cell surface APP (in all cells). The β -secretase cleavage site is located 28 residues from the plasma membrane luminal surface and the γ -secretase cleavage site is located in the transmembrane region. The *in vivo* processing of the β -secretase site is thought to be the rate limiting step in A β production (Sinha and Lieberburg, *Proc. Nat'l. Acad. Sci., USA* 96(4), 11049-11053, 1999) and as such is a favored target therapeutic target.

Recently, several groups of investigators have reported that a human aspartyl protease (Hu-Asp2) has an activity responsible for the processing of APP at the β -secretase cleavage site. Hu-Asp2 is a membrane-bound aspartyl protease (Yan *et al.*, *Nature* 402:533-536, 1999; Lin *et al.*, *Proc. Nat'l Acad. Sci., USA* 97(4):1456-1460, 2000; Vassar *et al.*, *Science*, 286:735-741, 1999). Aspartyl proteases such cathepsin D, Pepsin, renin, and viral aspartyl proteases comprise signature catalytic triplets ...Asp-Thr-Gly... and...Asp-Ser-Gly.... In Hu-Asp2, these signature catalytic triplets are harbored opposite to each other in the bilobed structure of Hu-Asp2. An important feature of Hu-Asp2 that is absent from other

known mammalian aspartyl proteases is a stretch of 27 amino acids located near the C-terminus that anchors Hu-Asp2 to the membrane and is essential for the enzyme function in cells.

To date, *in vitro* assays with recombinant Hu-Asp2 or Hu-Asp2
5 purified from brain have relied on peptide substrates comprising the β -secretase recognition sequence of the β -amyloid precursor sequence (*i.e.*, APP sequence), such as the APP Swedish mutation sequence ...EVNL-DAEFR... (SEQ ID NO:113) in which the hyphen denotes the point at which the peptide is cleaved by the β -secretase. The same approach has been used for β -secretase assays in cell lines
10 expressing Hu-Asp2.

There presently exists a need to identify compounds that may act as surrogates for the APP substrate of Hu-Asp2. Identifying such substrates facilitates new *in vitro* and model *in vivo* assays for identifying therapeutic agents that affect APP processing at the β -secretase site. Ultimately, the identification of such
15 substrates and assays will lead to advances in the identification of therapeutic compounds for the beneficial intervention of Alzheimer's Disease.

SUMMARY OF THE INVENTION

The present invention provides novel substrates, assays and methods
20 for conducting aspartyl protease assays. More particularly, one aspect of the present invention provides an isolated peptide comprising a sequence of at least four amino acids defined by formula $P_2P_1-P_1'P_2'$ wherein P_2 is a charged amino acid, a polar amino acid, or an aliphatic amino acid but is not an aromatic amino acid; P_1 is an aromatic amino acid or an aliphatic amino acid but not a polar amino acid or a
25 charged amino acid; P_1' is a charged amino acid, or aliphatic amino acid, or a polar amino acid but is not an aromatic amino acid; and P_2' is an uncharged aliphatic polar amino acid or an aromatic amino acid but not a charged amino acid; wherein the peptide is cleaved between P_1 and P_1' by a human aspartyl protease encoded by the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:3 and the peptide does
30 not comprise the corresponding $P_2P_1-P_1'P_2'$ portion of amino acid sequences

depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40.

5 In certain embodiments, the isolated peptide comprises an amino acid sequence defined by formula $P_2P_1-P_1'P_2'P_3'$, wherein P_3' is any amino acid, and wherein said peptide does not comprise the corresponding $P_2P_1-P_1'P_2'P_3'$ portion of amino acid sequences depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40.

10 In more specific embodiments, the isolated peptide comprises an amino acid sequence defined by formula $P_3P_2P_1-P_1'P_2'P_3'$, wherein P_3 is an uncharged polar amino acid, an uncharged aliphatic amino acid, or an aromatic amino acid, and wherein the the peptide does not comprise the corresponding $P_3P_2P_1-P_1'P_2'P_3'$ portion of amino acid sequences depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40.

15 In other embodiments, the isolated peptide comprises an amino acid sequence defined by formula $P_4P_3P_2P_1-P_1'P_2'P_3'$, wherein the P_4 is a charged amino acid, a polar amino acid or an aliphatic amino acid but not an aromatic amino acid and the peptide does not comprise the corresponding $P_4P_3P_2P_1-P_1'P_2'P_3'$ portion of amino acid sequences depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40. In other particular embodiments, the peptide further comprises an amino acid at position P_4' immediately to the carboxy-terminal position of P_3' , wherein the P_4' is any amino acid and wherein the peptide does not comprise the corresponding $P_3P_2P_1-P_1'P_2'P_3'P_4'$ portion of amino

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acid sequences depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40.

5 In particularly preferred embodiments, P_2 is an amino acid selected from the group consisting of N, L, K, S, G, T, D, A, Q and E.

 In preferred embodiments, P_1 is an amino acid selected from the group consisting of Y, L, M, Ile, F, and H.

 In preferred embodiments, define P_1' as an amino acid selected from
10 the group consisting of E, A, D, M, Q, S and G.

 In preferred embodiments, P_2' is an amino acid selected from the group consisting of V, A, N, T, L, F, and S.

 In preferred embodiments, P_3' is an amino acid selected from the group consisting of E, G, F, H, cysteic acid and S.

15 In preferred embodiments, P_3 is an amino acid selected from the group consisting of A, V, I, S, H, Y, T and F.

 In preferred embodiments, P_4 is an amino acid selected from the group consisting of E, G, I, D, T, cysteic acid and S.

 In preferred embodiments P_4' is an amino acid selected from the
20 group consisting of F, W, G, A, H, P, G, N, S, and E. Many highly preferred residues will be apparent from the detailed description.

 In preferred embodiments of the invention, the isolated peptide further comprises a first label. In specific embodiments, the peptide comprises a cysteic acid comprising a covalently attached label. Other preferred embodiments
25 contemplate that the peptide further comprises a second label. In this aspect of the invention, it is contemplated that the label may be any label commonly employed for the detection and/or quantification of a peptide. In specific embodiments, the peptide of the instant invention may comprise a detectable label and a quenching moiety that quenches a detectable property of the label when the label and quenching
30 moiety are in close proximity. More specifically, the quenching moiety and label

are selected such that cleavage of the peptide between P_1 and P_1' will separate the quenching moiety to permit detection of the label. Thus, in a preferred embodiment, the quenching moiety and label are attached to the peptides on opposite sides of the P_1 - P_1' cleavage site.

5 In preferred embodiments of the present invention, the invention provides a genus of peptides that are cleaved by the human aspartyl protease Hu-Asp2 at a rate greater than the rate of cleavage of a polypeptide comprising the human APP β -secretase cleavage sequence: SEVKM-DAEFR (SEQ ID NO:20). Still more preferred embodiments provide a genus of peptides cleaved by the human
10 aspartyl protease at a rate greater than the rate of cleavage of a polypeptide comprising the human APP Swedish KM~NL mutation, β -secretase cleavage sequence SEVNL-DAEFR (SEQ ID NO:19).

Particularly preferred peptides of the present invention include but are not limited to those comprising any one of the sequences set forth in SEQ ID
15 NO:5; SEQ ID NO:6; SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:9; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:12; SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15; SEQ ID NO:16; SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:120; SEQ ID NO:133; SEQ ID NO:134; SEQ ID NO:135; SEQ ID NO:136; SEQ ID NO:137; SEQ ID NO:138; SEQ ID NO:141; SEQ ID NO:143; SEQ ID NO:144;
20 SEQ ID NO:145; SEQ ID NO:147; SEQ ID NO:148; SEQ ID NO:149; SEQ ID NO:150; SEQ ID NO:151; SEQ ID NO:152; SEQ ID NO:153; SEQ ID NO:154; SEQ ID NO:155; SEQ ID NO:156; SEQ ID NO:157; SEQ ID NO:158; SEQ ID NO:159; SEQ ID NO:160; SEQ ID NO:161; SEQ ID NO:162; SEQ ID NO:163; SEQ ID NO:164; SEQ ID NO:165; SEQ ID NO:166; SEQ ID NO:167; SEQ ID
25 NO:168; SEQ ID NO:169; SEQ ID NO:190; SEQ ID NO:191; SEQ ID NO:192 and SEQ ID NO:193.

An additional aspect of the present invention relates to fusion polypeptides comprising the amino acid sequence of a novel β -secretase cleavage site described by one of the peptides of the instant invention and an additional amino
30 acid sequence. More particularly, the invention contemplates a polypeptide

comprising a peptide of the instant invention and further comprising a transmembrane domain amino acid sequence. In specific embodiments, the peptide containing the β -secretase cleavage site is N-terminal to the transmembrane domain, but C-terminal also is contemplated. In certain embodiments, the peptide and the transmembrane domain may be separated by a linker. More specifically, the linker may be a peptide linker comprising about 20 to about 40 amino acids. In particularly preferred embodiments, the transmembrane domain anchors the polypeptide to an intracellular membrane selected from the group consisting of the Golgi or the endoplasmic reticulum. As such, the transmembrane domain comprises the transmembrane domain amino acid sequence of a protein that is anchored to the Golgi or ER membrane of cells that express such a protein. In preferred embodiments, the fusion polypeptide comprises a transmembrane domain selected from the group consisting of the transmembrane domain of galactosyltransferase, the transmembrane domain of sialyl transferase; the transmembrane domain of human aspartyl transferase 1; the transmembrane domain of human aspartyl transferase 2; the transmembrane domain of syntaxin 6; the transmembrane domain of acetyl glucosaminyl transferase; and the transmembrane domain of APP.

In preferred embodiments of the invention the fusion protein may further comprise a reporter protein amino acid sequence in addition to the peptide sequence and the transmembrane sequence. The reporter sequence preferably provides a detectable and quantifiable characteristic (*e.g.* an optical characteristic) or is an enzyme that catalyzes a substrate into a product, wherein the substrate or product provides a detectable and quantifiable characteristic. Also contemplated are, fusion polypeptides comprising a reporter protein amino acid sequence and a β -secretase cleavage site containing peptide sequence of the present invention. In preferred aspects of the invention, the reporter protein is selected from the group consisting of luciferase; alkaline phosphatase; β -galactosidase; β -glucuronidase; green fluorescent protein; and chloramphenicol acetyl transferase.

In certain aspects, the present invention contemplates a mutant or derivative APP molecule in which the natural β -secretase cleavage site of wild-type

APP has been modified to contain a b-secretase cleavage site of one of the substrates of the present invention. Such a mutant or derivative APP may be generated by site directed mutagenesis or by peptide synthesis as described herein.

Additional aspects of the invention contemplate a polynucleotide
5 comprising a nucleotide sequences that encodes a fusion polypeptide of the present invention. Other embodiments contemplate a polynucleotide comprising a nucleotide sequence that encodes a peptide substrate of the present invention. Also contemplated herein is a vector comprising any of the polynucleotides of the present invention. In preferred embodiments, the vector comprises a polynucleotide
10 encoding a peptide or a fusion polypeptide of the present invention, wherein the polynucleotide is operably linked to a promoter to promote expression of the fusion protein encoded by the polynucleotide in a host cell. Other embodiments contemplate a host cell transformed or transfected with a polynucleotide or vector described by the present invention. In a preferred embodiment, the host cell is co-
15 transfected with the polynucleotide/vector and a polynucleotide/vector encoding Hu-Asp2.

Also described herein is a method of producing a substrate for a β -secretase assay comprising growing a host cell transformed or transfected with a vector of the present invention in a manner that permits the expression of the
20 polypeptide. Additional embodiments of this aspect of the invention may further comprise purifying the polypeptide. In preferred embodiments, the host cell is selected from the group consisting of a mammalian host cell, a bacterial host cell and a yeast host cell.

Also contemplated herein are transgenic animals comprising an APP
25 mutant having as β -secretase cleavage one of the peptides of the present invention.

In a further aspect, the present invention provides a method for assaying for modulators of β -secretase activity, comprising the steps of contacting a first composition with a second composition both in the presence and in the absence of a putative modulator compound, wherein the first composition comprises a
30 mammalian β -secretase polypeptide or biologically active fragment thereof, and

wherein the second composition comprises a substrate, wherein the substrate comprises a peptide or a fusion polypeptide of the present invention. In preferred embodiments, the method further comprises measuring cleavage of the substrate peptide in the presence and in the absence of the putative modulator compound; and
5 identifying modulators of β -secretase activity from a difference in cleavage in the presence versus in the absence of the putative modulator compound, wherein a modulator that is a β -secretase antagonist reduces such cleavage and a modulator that is a β -secretase agonist increases such cleavage. In specific embodiments, the first composition may comprise a purified human Asp2 polypeptide. In other
10 embodiments, the first composition may comprise a soluble fragment of a human Asp2 polypeptide that retains Asp2 β -secretase activity. More particularly, the soluble fragment may be a fragment lacking an Asp2 transmembrane domain. In preferred embodiments, the β -secretase polypeptide of the first composition comprises a polypeptide purified and isolated from a cell transformed or transfected
15 with a polynucleotide comprising a nucleotide sequence that encodes the β -secretase polypeptide.

In other specific embodiments, the first composition comprises in a cell transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes the β -secretase polypeptide, and the measuring step
20 comprises measuring APP processing activity of the cell.

Further aspects of the invention contemplate treating Alzheimer's Disease with an agent identified as an inhibitor of Hu-Asp2 according to the methods of the present invention. Preferred aspects further contemplate a β -secretase modulator identified according to the methods of the present invention.
25 Specifically contemplated are methods of inhibiting β -secretase activity *in vivo* comprising a step of administering a modulator identified by the present invention, wherein the modulator is a β -secretase antagonist, to a mammal in an amount effective to inhibit β -secretase in cells of the mammal.

Also described herein is a pharmaceutical composition comprising a
30 β -secretase modulator identified by the present invention and a pharmaceutically

acceptable carrier. Further aspects of the invention describe a method of treating a disease or condition characterized by an abnormal β -secretase activity comprising administering to a subject in need of treatment a pharmaceutical composition as described above. Other embodiments describe the use of a modulator identified according to the present invention in the manufacture of a medicament for the treatment of Alzheimer's Disease.

In a related aspect, the invention provides a container containing a composition comprising a modulator identified according to the present invention, the container containing a label describing the indication of the modulator for treating Alzheimer's Disease. Preferably, the composition further comprises a pharmaceutical carrier. Most preferably, the composition is a unit dose formulation.

Additional methods are described for identifying agents that inhibit the activity of human Asp2 aspartyl protease (Hu-Asp2), comprising the steps of contacting a peptide or a fusion polypeptide of the invention and a composition comprising an Hu-Asp2 activity in the presence and absence of a test agent; determining the cleavage of the peptide or polypeptide between the P_1 and P_1' by the Hu-Asp2 in the presence and absence of the test agent; and comparing the cleavage activity of the Hu-Asp2 in the presence of the test agent to the activity in the absence of the test agent to identify an agent that inhibits the cleavage by the Hu-Asp2, wherein reduced activity in the presence of the test agent identifies an agent that inhibits Hu-Asp2 activity. In preferred embodiments, the Hu-Asp2 is a recombinant Hu-Asp2 purified and isolated from a cell transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes Hu-Asp2. In other embodiments, the Hu-Asp2 is expressed in a cell, wherein the contacting comprises growing the cell in the presence and absence of the test agent, and the determining step comprises measuring cleavage of the peptide or fusion protein. In specific embodiments, the cell further comprises a polynucleotide encoding the polypeptide, and wherein the contacting step comprises growing the cell under conditions in which the cell expresses the polypeptide. In preferred embodiments,

the cell is a human embryonic kidney cell line 293 cell and a mouse Neuro-2a neuroblastoma cell line. In other preferred embodiments, the nucleotide sequence is selected from the group consisting of a nucleotide sequence encoding the Hu-Asp2(a) amino acid sequence set forth in SEQ ID NO: 2; a nucleotide sequence
5 encoding the Hu-Asp2(b) amino acid sequence set forth in SEQ ID NO: 4; a nucleotide sequence encoding a fragment of Hu-Asp2(a) (SEQ ID NO: 2) or Hu-Asp2(b) (SEQ ID NO: 4), wherein the fragment exhibits aspartyl protease activity characteristic of Hu-Asp2(a) or Hu-Asp2(b); and a nucleotide sequence of a polynucleotide that hybridizes under stringent hybridization conditions to a Hu-
10 Asp2-encoding polynucleotide selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 3.

An additional aspect describes a method for identifying agents that modulate the activity of Asp2 aspartyl protease, comprising the steps of contacting an Asp2 aspartyl protease and a peptide or fusion polypeptide of the present
15 invention in the presence and absence of a test agent, wherein the Asp2 aspartyl protease is encoded by a nucleic acid molecule that hybridizes under stringent hybridization conditions to a Hu-Asp2-encoding polynucleotide selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 3; determining the cleavage of the peptide or fusion protein between the P_1 and the P_1' site by the Asp2 in the
20 presence and absence of the test agent; and comparing the cleavage activity of the Asp2 in the presence of the test agent to the cleavage activity in the absence of the agent to identify agents that modulate the activity of the polypeptide, wherein a modulator that is an Asp2 inhibitor reduces the cleavage and a modulator that is an Asp2 agonist increases the cleavage. In preferred embodiments, the method further
25 comprises the step of treating Alzheimer's Disease with an agent identified as an inhibitor of Hu-Asp2 according to the instant invention.

Also described is a method for identifying agents that inhibit the activity of human Asp2 aspartyl protease (Hu-Asp2), comprising the steps of growing a cell in the presence and absence of a test agent, wherein the cell
30 expresses a Hu-Asp2 or mouse Asp2 and expresses a protein comprising a peptide

or fusion polypeptide of the present invention; determining the cleavage of the protein at the site between the P_1 and P_1' in the cell in the presence and absence of the test agent; and comparing the cleavage activity in the presence of the test agent to the cleavage activity in the absence of the test agent to identify an agent that inhibits the activity of Hu-Asp2, wherein reduced cleavage activity in the presence of the test agent identifies an agent that inhibits Hu-Asp2 activity. In preferred embodiments, the host cell either expresses endogenous Asp2 or has been transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes a Hu-Asp2, wherein the nucleotide sequence is selected from the group consisting of a nucleotide sequence encoding the Hu-Asp2(a) amino acid sequence set forth in SEQ ID NO: 2; a nucleotide sequence encoding the Hu-Asp2(b) amino acid sequence set forth in SEQ ID NO: 4; a nucleotide sequence encoding a fragment of Hu-Asp2(a) (SEQ ID NO: 2) or Hu-Asp2(b) (SEQ ID NO: 4), wherein the fragment exhibits aspartyl protease activity characteristic of Hu-Asp2(a) or Hu-Asp2(b); and a nucleotide sequence of a polynucleotide that hybridizes under stringent hybridization conditions to a Hu-Asp2-encoding polynucleotide selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 3. The invention also contemplates the use of an agent identified as an inhibitor of Hu-Asp2 according the present invention in the manufacture of a medicament for the treatment of Alzheimer's Disease.

The preceding paragraphs describe methods for identifying modulators of aspartyl protease activity using peptide substrates of the invention, or using polypeptides comprising the peptides. With respect to each of those methods, a preferred embodiment includes an additional step of synthesizing more of a modulator that has been identified as an aspartyl protease inhibitor. In a preferred variation, the method further comprises synthesizing a composition comprising the modulator in a pharmaceutically acceptable diluent, adjuvant, or carrier. The methods optionally comprise still a further step of administering the composition to a mammal to inhibit aspartyl protease activity in the mammal.

Additionally, the invention contemplates kits for performing a β -

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secretase assay comprising a β -secretase substrate peptide of the present invention packaged with a β -secretase enzyme. In specific embodiments, the β -secretase substrate may be a fusion polypeptide of the present invention. Further
5 embodiments contemplate that the kits may comprise reagents for detecting the cleavage of the peptide or fusion protein. The invention also describes other preferred peptides which comprise a sequence of at least 10 amino acids having the sequence SEISY-EVEFR (SEQ ID NO:152). Also contemplated are peptides which comprise 3, 4, 5, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acids immediately to the carboxy-terminal of SEISY-EVEFR (SEQ
10 ID NO:152) and/or immediately to the amino-terminal of SEISY-EVEFR (SEQ ID NO:152). Preferred peptides may be 10, 13, 15, 16, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200 amino acids or longer.

Additional features and variations of the invention will be apparent to
15 those skilled in the art from the entirety of this application, including the drawing and detailed description, and all such features are intended as aspects of the invention. Likewise, features of the invention described herein can be re-combined into additional embodiments that also are intended as aspects of the invention, irrespective of whether the combination of features is specifically mentioned above
20 as an aspect or embodiment of the invention. Also, only such limitations which are described herein as critical to the invention should be viewed as such; variations of the invention lacking limitations which have not been described herein as critical are intended as aspects of the invention.

In addition to the foregoing, the invention includes, as an additional
25 aspect, all embodiments of the invention narrower in scope in any way than the variations specifically mentioned above. Although the applicant(s) invented the full scope of the claims appended hereto, the claims appended hereto are not intended to encompass within their scope the prior art work of others. Therefore, in the event that statutory prior art within the scope of a claim is brought to the attention of the
30 applicants by a Patent Office or other entity or individual, the applicant(s) reserve the right to exercise amendment rights under applicable patent laws to redefine the

subject matter of such a claim to specifically exclude such statutory prior art or obvious variations of statutory prior art from the scope of such a claim. Variations of the invention defined by such amended claims also are intended as aspects of the invention. The detailed description presented below, while providing preferred
5 embodiments of the invention, is intended to be illustrative only since changes and modification within the scope of the invention will be possible whilst still providing an embodiment that is within the spirit of the invention as a whole.

BRIEF DESCRIPTION OF THE DRAWINGS

10 The following drawing forms part of the present specification and is included to further demonstrate aspects of the present invention. The invention may be better understood by reference to the drawing in combination with the detailed description of the specific embodiments presented herein.

15 FIG. 1A through FIG. 1D depicts a contiguous, complete sequence of an exemplified fusion polypeptide (SEQ ID NO:128) of the present invention and the nucleotide sequence encoding the polypeptide (SEQ ID NO:127). The entire coding sequence for secreted alkaline phosphatase from MLLL (SEQ ID NO:44)... to DAAHPG (SEQ ID NO:45) is shown in normal font. Sequences derived from insulin β -chain with the modified β -secretase cleavage site are italicized. The transmembrane domain from Asp2 454-477 is underlined. DYKDDDK (SEQ ID
20 NO:114) is a flag tag sequence.

FIG. 2 depicts proposed chimeras as substrates for Asp2 in cell based assays. The portion designated "ecto" refers to ectodomain, the portion designated "endo" refers to endodomain and the portion designated "memb" refers to the membrane domain of the chimeric protein respectively.

25 FIG. 3 contains images depicting Western blot analysis of APP processing by β -secretase. A stable HEK-293 cell line that expresses higher levels of Asp2 was transfected with either wt-APP or different mutant APP DNA constructs. After transfection for 48 hours, cell extracts were analyzed by Western analysis. The antibody C8 which recognizes the C-terminus of human APP was
30 used for detecting full-length APP and its processing products. The β -secretase activity was judges by the production of CTF99 or the ratio of CTF99 over CTF83.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Alzheimer's disease is a leading age-related disorder associated with progressive dementia and pathology characterized by cortical atrophy and deposition of senile plaques and neurofibrillary tangles. A primary component of the plaques is the 40-42 amino acid long peptide, A β derived from a region of APP adjacent to and containing a portion of the transmembrane domain of the full length APP. This pathogenic peptide is generated as a result of sequential processing due to β - and γ -secretases activities. While there has been extensive hunt for the identity of these enzymes, the exact identity of the protein has remained elusive. Recently, however, significant evidence has accumulated to suggest that Hu-Asp2 may be a likely candidate for the β -secretase activity. There present invention provides compounds, molecules or substrates that may be cleaved by Hu-Asp2 and act as surrogates for the APP substrate. Methods and compositions for making and using these compounds are provided in further detail below.

I. Novel Peptide Substrates for Hu-Asp2

The present invention provides substrates for Hu-Asp2. Initially, a number of peptides were tested as potential substrates of Hu-Asp2. The peptides:

5 AcGSESMDSGISL-DNKW (SEQ ID NO:115),
 WKKGAIIGL-MVGGVVKK (SEQ ID NO:116),
 ANL-STFAQPRR (SEQ ID NO:117),
 YRYQSHDYAF-SSVEKLLHLGGC (SEQ ID NO:118),
 YRYQSHDY-AFSSVEKLLHLGGC (SEQ ID NO:119)

10 are cleaved between the two amino acids separated by a hyphen.

 Also (His)₆Ubiquitin and the fusion protein (His)₆Ubiquitin-CTAPIII are cleaved at the C-terminal side of a Leu residue in the sequence

 ...KTITL-EVEPS.... (SEQ ID NO:120)

 However, all these substrates are cleaved less efficiently than the
15 peptide corresponding to the Swedish mutant form of APP: SEVNL-DAEFR (SEQ ID NO:19). Interestingly, the oxidized insulin B chain is a substrate of Asp2 and is cleaved at a rate comparable to SEVNL-DAEFR (SEQ ID NO:19). The cleavage site for the insulin B chain is between Tyr and Leu in the sequence
 VEALY-LVC(SO₃)GER (SEQ ID NO:121). An additional peptide with a Glu in
20 place of cysteic acid (VEALY-LVEGER (SEQ ID NO:122) also was tested and found to be cleaved by the Hu-Asp2. Using these observations, the inventors conducted further comparisons of the above Hu-Asp2 substrates with additional known Hu-Asp2 substrates to elucidated information regarding specific amino acid occupancy at and around the cleavage site.

25 Conventional abbreviations for amino acids are used herein, as follows:

Alanine, Ala, A; Arginine, Arg, R; Asparagine, Asn, N; Aspartic acid, Asp, D; Cysteine, Cys, C; Glutamine, Gln, Q; Glutamic Acid, Glu, E; Glycine, Gly, G; Histidine, His, H; Isoleucine, Ile, I; Leucine, Leu, L; Lysine, Lys, K;

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Methionine, Met, M; Phenylalanine, Phe, F; Proline, Pro, P; Serine, Ser, S; Threonine, Thr, T; Tryptophan, Trp, W; Tyrosine, Tyr, Y; Valine, Val, V; Aspartic acid or Asparagine, Asx, B; Glutamic acid or Glutamine, Glx, Z; Norleucine, Nle; Acetyl-glycine (Ac)G; Any amino acid, Xaa, X. Additional
 5 modified amino acids also may be used as described herein. For example, C(SO₃) refers to cysteic acid.

The peptides depicted in Table 1 comprise a hyphen to indicate the estimated cleavage site for Hu-Asp2.

10	Peptide Sequence	Origin/identity	Sequence identifier
	SEVNL-DAEFR	β -secretase, A β Swedish Mutant Sequence	SEQ ID NO:19
	SEVKM-DAEFR	β -secretase, A β Normal Sequence	SEQ ID NO:20
	SEVN(Nle)-DAEFR	β -secretase, A β Swedish Mutant L \rightarrow Nle	SEQ ID NO:21
	(Ac)GSESMDSGISL-DNKW	Casp-3 prosegment	SEQ ID NO:22
15	WKKGAIIIGL-MVGGVVKK	δ -cleavage on A β	SEQ ID NO:23
	ANL-STFAQPRR	Novel Sequence	SEQ ID NO:24
EFRHDSGY-EVHHQKLVFFAE....	cleavage on Ab	SEQ ID NO:25

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LTGKTITL-EVEPSDTL.....	(His) ₆ Ubiquitin-CT APIII sequence	SEQ ID NO:26
	FVNQHLC _{ox} GSHLVEALY-LVC _{ox} GER GFFYTPKA	oxidized Insulin B chain sequence	SEQ ID NO:27
5	GIVEQCoxC _{ox} ASVC _{ox} SLY-QLENYC _{ox} N	oxidized Insulin A chain sequence	SEQ ID NO:28
	YRYQSHDY-AFSSVEKLLHALGGC	Novel Sequence of the present invention	SEQ ID NO:29
	YRYQSHDYAF-SSVEKLLHALGGC		SEQ ID NO:30
	LVNM-AEGD	PS1	SEQ ID NO:31
	RGSM-AGVL	M2-Pro	SEQ ID NO:32
10	GTQH-GIRL	M2-Pro	SEQ ID NO:33
	SSNF-AVGA	M2	SEQ ID NO:34
	GLAY-AEIA	M2	SEQ ID NO:35
	HLCG-SHLV	Oxidized Insulin B-chain	SEQ ID NO:36
	CGER-GFFY	Oxidized Insulin B-chain	SEQ ID NO:37
15	GVLL-SRK	Notch	SEQ ID NO:38
	VGS-GVLL	Notch	SEQ ID NO:39

V-GSGV	Notch	SEQ ID NO:40
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As used herein throughout, C_{ox} and C(SO₃) refer to oxidized cysteine otherwise referred to herein as cysteic acid, these terms are used interchangeably herein and in the art. It should be noted that wherever cysteic acid is used at a particular residue in peptide substrates of the present invention, additional substrates that comprise cysteine at that residue also are contemplated. In preferred embodiments, the cysteine in SEQ ID NO:36 and SEQ ID NO:37 is oxidized to cysteic acid.

The peptides of the present invention are described using the nomenclature by Schechter and Berger (*Biochem. Biophys. Res. Commun.* 27:157(1967) and *Biochem. Biophys. Res. Commun.* 32:898 (1968), in which the amino acid residues in the peptide substrate that undergo the cleavage are defined as P₁ . . . P_n moving from the scissile bond toward the N-terminus and P₁' . . . P_n' moving from the scissile bond toward the C-terminus. Therefore, the scissile bond is between the P₁ and the P₁' residue of the peptide subunits and is denoted herein throughout with a hyphen between the P₁ and the P₁'.

In the peptides of the present invention that were effective Hu-Asp2 substrates, Tyr/Phe and Leu were the most abundant amino acids at the P₁ site; Asn appeared several times at the P₂ site; Glu, Asp, and Ala, were prominent in the P₁'; Val occurred frequently in the P₂'; the sequence Glu-Val-Glu appeared at the P₁' P₂' P₃' of ubiquitin, another Asp2 substrate; other positions did not show any obvious preferences. These observations were used to make amino acid substitutions around the cleavage site of the oxidized insulin B-chain, specifically at P₂-P₃'.

The peptides listed in Table 2 were designed, synthesized, and tested for activity as substrates for Hu-Asp2. 200μM of each test substrate was incubated with 210nM Hu-Asp2 enzyme at pH 4.5 at 37°C for 3 hours. Of course it should be understood that these are merely exemplary assay conditions, and those of skill in the art will be able to vary these conditions and yet still provide appropriate Hu-Asp2 activity. For example, it is envisioned that the assays may be conducted at a pH range of from between about 4.0 to about 7.0. The amount of enzyme added to

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a given reaction mixture may also be varied and those of skill in the art will be able to perform assays defining the optimal enzyme concentrations necessary for a given assay, as such 150nm; 200nm; 250nm; 300nm or more or less enzyme may be added. Substrate concentration in those also may be varied such that an assay may
5 use 100; 150 μ M, 200 μ M, 250 μ M, 300 μ M, 350 μ M or more or less of any given substrate. It may be that one substrate produces an optimum activity at one concentration whereas another substrate is needed to be present at a different concentration to produce an optimum cleavage rate. However, such optimizations of enzyme assay conditions is well within the skill of those in the art and will not
10 require undue experimentation.

In Table 2 below, substrates that produced activity are indicated by “+” signs in which the greater the activity, the more + signs are provided. Those substrates that were not cleaved by the Hu-Asp2 are indicated by a “-” sign.

Table 2

Peptide Sequence	Activity	Sequence Identifier
KVEALY-LV(SO3-)-GER	-	SEQ ID NO:41
WRRVEALY-LVEGERK	++	SEQ ID NO:42
KVEANY-LVEGERKK	+	SEQ ID NO:43
KVEANY-EVEGERKK	++++	SEQ ID NO:5
KVEANY-AVEGERKK	++++	SEQ ID NO:7
KVEANY-DVEGERKK	+	SEQ ID NO:46
KVEANL-AVEGERKK	+	SEQ ID NO:47
KVEALY-AVEGERKK	+	SEQ ID NO:48

From the above list of putative substrates, KVEANY-EVEGERKK (SEQ ID NO:5) was selected for further studies. Specifically, a Cys was inserted between R and KK to yield the peptide KVEANY-EVEGERCKK (SEQ ID NO:6). This peptide was a good substrate for Hu-Asp2. In additional steps this peptide was N-terminally biotinylated, and made fluorescent by the covalent attachment of oregon green at the Cys residue. The resulting compound, Biotin-KVEANY-EVEGERC(oregon green)KK (SEQ ID NO:49), was tested in the following conditions: 10 μ M substrate, 50nM enzyme at 37 °C. The reaction was allowed to proceed for 2hrs and samples withdrawn at several times. The results showed that 20%, 37%, 57%, and 82% cleavage occurred after 15, 30, 60, and 120 minutes, respectively. Mass spectrometry analysis showed that cleavage had occurred between Tyr and Glu only. It was shown that the new biotinylated fluorescent peptide had Vmax and Km values that were at least double the value obtained for a previous biotinylated fluorescent substrate derived from the Swedish Mutant peptide.

The EVE and AVE sequences, that appear in positions P₁'-P₃' of SEQ ID NO:5 and SEQ ID NO:7, respectively (Table 2), were selected for

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additional modification. As in the case of development of the fluorescent substrate Biotin-KVEANY-EVEGERC(Oregon Green)KK (SEQ ID NO:49), the EVE sequence was selected as a starting point for modifications. Specifically, the EVE sequence in the context of the decapeptide KTITL-↓-EVEPS (SEQ ID NO:120) was selected, because the amino acid sequence in this decapeptide is an exact mimic of a stretch of ten amino acids encompassing the cleavage site L-↓-E found in ubiquitin (see SEQ ID NO:26 in Table 1).

Table 3. Substrate optimization for Asp2 based upon a cleavage site in ubiquitin

Peptide	Peptide Sequence	(SEQ ID NO)	Relative rate of Cleavage+
APP-Sw	SEVNL- ↓-DAEFR	(SEQ ID NO:19)	100
APP-Wildtype	SEVKM-↓-DAEFR(SEQ ID NO:20)		2.1*
Ubiquitin (Pep 1)	KTITL-↓-EVEPS	(SEQ ID NO:120)	13.1
Peptide 2	KTINL-↓-EVEPS	(SEQ ID NO:133)	92.0
Peptide 3	KTIN _{nle} -↓-EVEPS	(SEQ ID NO:134)	92.6
Peptide 4	KTIN _{nle} -↓-EVDPS	(SEQ ID NO:135)	93.6
Peptide 5	KTIN _{nle} -↓-DVDPS	(SEQ ID NO:136)	12.2
Peptide 6	KTISL- ↓-DVEPS	(SEQ ID NO:137)	69.0
Peptide 7	KTISL- ↓-DVDPS	(SEQ ID NO:138)	20.5

nle=norleucine

+ Assay conditions were 200 nM enzyme, and 200 μM substrate, 300 mM Na-acetate, 4% DMSO, at pH 4.5, 37 °C for 4hr.

* 24hr reaction

In addition to modifications at the P₁'-P₃' (EVE) sites, modifications were also made at the P₂-P₁ sites. Six variants of KTITL-↓-EVEPS (SEQ ID NO:120) were constructed and compared their activities as substrates of Asp2 to the

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activities shown by the Swedish mutation peptide, SEQ ID NO:19, and wild type peptide, SEQ ID NO:20 (Table 3). The ubiquitin-derived peptide having the sequence of SEQ ID NO:120, was six times better than wild type peptide, SEQ ID NO:20, but had only about 13% of the activity shown by the Swedish mutation, SEQ ID NO:19 (Table 3). However, substitution of the P₂ Thr with Asn generated peptide having the sequence of SEQ ID NO:133 with activity similar to that of the Swedish mutant, SEQ ID NO:19. Substitution of Leu in position P₁ of peptide #2 SEQ ID NO:133 with the unnatural amino acid norleucine (peptide #3 SEQ ID NO:134) gave activity similar to that of the Swedish mutation. Also the substitution of Glu of peptide #3 SEQ ID NO:134 in P₃' with Asp (peptide #4; SEQ ID NO:135) was very well tolerated. However, mutation of the two Glu residues that are at positions P₁' and P₃' in peptide #3 (SEQ ID NO:134), with two Asp residues, to give peptide #5 (SEQ ID NO:136), reduced the activity about 10 fold. Peptide #1, SEQ ID NO:120 was modified by putting a serine residue in P₂ and an Asp in position P₁' to give KTISL-↓-DVEPS (peptide #6; SEQ ID NO:137). This double substitution was well tolerated, giving about 35 times more activity than wild-type peptide and 69% of the activity of Swedish mutation SEQ ID NO:19. However, when an Asp was put in place of Glu in the P₃' position of peptide #6 (SEQ ID NO:137), to give peptide #7 (SEQ ID NO:138), activity dropped substantially. As observed with peptide #5 (SEQ ID NO:136), the Glu residues at positions P₁' and P₃' are highly preferred to Asp residues in the same positions.

Optimization of the Asp2 substrate described above leaves an Asn at P₂ (Table 2 and 3). This is acceptable for an *in vitro* assays because optimization was done within the context of insulin B-chain and ubiquitin. However, in some cases, it will be desirable to develop a cell-based assay with minimal disruption of the APP sequence. To this end, studies involving variations to the sequences of insulin B-chain and ubiquitin have yielded a great deal of information about the effects of amino acids at and near the β-secretase cleavage site of Asp2 and their effects on APP processing by this enzyme. This information can be used to modify the P₂-P₂' residues in the sequences SEVKM-↓-DAEFR (SEQ ID NO:20)/ SEVNL-↓-DAEFR (SEQ ID NO:19) (wild type/Swedish mutation) and produce minimally

altered APP forms that are highly susceptible to β -secretase cleavage. Such substrates will be useful in cell-based assays for APP processing, A β production, and β -secretase modulation, as well as any other assay that may employ a wild-type or mutant APP.

5 Using the above principles, the inventors further explored the residues at the P₂ position of APP to discover that serine is a good replacement for Lys/Asn in the Asp2 substrates of the present invention. Thus, in particularly preferred embodiments, the amino acid at position P₂ in the substrates of the present invention is serine. Accordingly, replacement of the four amino acids surrounding
10 the β -secretase cleavage site in APP, *i.e.* ...KM-↓-DA....(SEQ ID NO:139), with ...SY-↓-EV....(SEQ ID NO:140) produced the peptide SEVSY-↓-EVEFR (SEQ ID NO:141) that was hydrolyzed by Asp2 at a rate that was 70-fold greater than that seen for wild type.

Thus, the inventors have produced substrates superior to the Swedish
15 mutation peptide (with respect to β -secretase cleavage) by changing the four amino acids ...NL-↓-DA....(SEQ ID NO:142) which encompass the β -secretase cleavage site in APP Swedish mutation to ...SY-↓-EV....(SEQ ID NO:141). This discovery provides the means for producing a minimally altered APP that is highly susceptible to β -secretase cleavage and therefore serves as the basis for improved cell based
20 assays for testing for inhibitors of Asp2.

Table 4. Substrate optimization for Asp2 incorporating all available information

Peptide Sequence (Sequence Listing No.)	Relative rate of Cleavage ⁺
25 SEVNL-↓-DAEFR (SEQ ID NO:19)	100.0
SEVKM-↓-DAEFR (SEQ ID NO:20)	2.1*
SEVSY-↓-DAEFR (SEQ ID NO:143)	18.3
SEVSY-↓-EAEFR (SEQ ID NO:144)	77.1
SEVSY-↓-EVEFR (SEQ ID NO:141)	141.8

30 ⁺ Assay conditions were 200 nM enzyme, and 200 μ M substrate, pH 4.5, 37 °C for 1hr.

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* 24hr reaction

In Table 4, the activity of Asp2 with three newly synthesized compounds, wild type, and the Swedish mutant are compared. SEVSY-↓-DAEFR (SEQ ID NO:143) activity has increased about 9 fold over wild-type, but is still about five times less than that of the Swedish mutation SEQ ID NO:19. The substitution of the triplet KM-↓-D with SY-↓-E to give SEVSY-↓-EAEFR (SEQ ID NO:144) produced improvement of activity of the order of 40-fold over wild-type and had activity comparable to that of the Swedish mutant. A near 70-fold increase of activity over wild type peptide and a 1.4-fold increase over Swedish mutant is obtained when the four amino acids, KM-↓-DA (SEQ ID NO:139), at the active site of the wild type peptide, are substituted with SY-↓-EV (SEQ ID NO:140) to give the sequence SEVSY-↓-EVEFR (SEQ ID NO:141).

In addition, the inventors generated additional substrates to further improve on substrate SEVSY-↓-EVEFR (SEQ ID NO:141) for Asp2 cleavage potential. The results are summarized in Table 5. In one strategy, SEVSY-↓-EVEFR (SEQ ID NO:141) was N-terminally extended, specifically by 5-, 10-, and 15-residues. All of the amino acids comprised in these extensions are from the APP natural sequence.

Table 5. Substrate optimization for Asp2

Peptide Sequence (Sequence Listing Entry)	Relative rate of cleavage	Cleavage Activity $\mu\text{mol/min/mg}$
TRPGSGLTNIKTEEISEVSY-EVEFR (SEQ ID NO:145)	840	1.35
GLTNIKTEEISEVSY-EVEFR (SEQ ID NO:146)	ND	ND
KTEEISEVSY-EVEFR (SEQ ID NO:147)	360	0.58
SEVSY-EVEFR (SEQ ID NO:141)	100	0.16
TEVSY-EVEFR (SEQ ID NO:148)	160	0.27
SEVDY-EVEFR (SEQ ID NO:149)	240	0.38

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TEVDY-EVEFR (SEQ ID NO:150)	230	0.37
TEIDY-EVEFR (SEQ ID NO:151)	390	0.62
SEISY-EVEFR (SEQ ID NO:152)	500	0.80
SEIDY-EVEFR (SEQ ID NO:153)	430	0.69

5 Assay conditions were 2 nM enzyme, and 50 μ M substrate, 25 mM Sodium acetate, pH 4.4, 37 °C for 2hr.

 Interestingly, TRPGSGLTNIKTEEISEVSY-EVEFR (SEQ ID NO:145), is cleaved at a rate 8.4 times faster than SEVSY-↓-EVEFR (SEQ ID NO:141), Table 5. The peptide KTEEISEVSY-EVEFR (SEQ ID NO:147) that is only
10 5 residues longer than SEVSY-↓-EVEFR (SEQ ID NO:141), is cleaved at a rate 3.6 times faster than the latter peptide (Table 5). In the second strategy, the inventors modified SEVSY-↓-EVEFR (SEQ ID NO:141) on the P side, specifically at the P₅, P₃, and P₁ positions to augment activity towards Asp2. The inventors used this
15 strategy to yield substrates that achieved levels of activity higher than the activity observed with SEVSY-↓-EVEFR (SEQ ID NO:141) (Table 5). The particularly good substrate of the present invention is SEISY-EVEFR (SEQ ID NO:152), where Ile is taking the place of the P₃ Val in SEVSY-↓-EVEFR (SEQ ID NO:141). The substitution of Val with Ile resulted in a 5-fold increase in activity.

20 The amino acid Ile in position P₃ corresponds with the ubiquitin cleavage site (see Table 3). Based on the results of Table 5, the inventors expect that N-terminus extension of SEISY-EVEFR (SEQ ID NO:152) by, *e.g.*, 5 to 15 residues, will result in a substantial increase in activity. Examples of such extended peptides include but are not limited to SEISY-EVEFRXKK (SEQ ID NO:154);
25 KTEEISEISY-EVEFRXKK (SEQ ID NO:155); GLTNIKTEEISEISY-EVEFRXKK (SEQ ID NO:156); TRPGSGLTNIKTEEISEISY-EVEFRXKK (SEQ ID NO:157); Biotin-SEISY-EVEFRXKK (SEQ ID NO:158); Biotin-KTEEISEISY-EVEFRXKK (SEQ ID NO:159); Biotin-GLTNIKTEEISEISY-EVEFRXKK (SEQ ID NO:160); Biotin-TRPGSGLTNIKTEEISEISY-EVEFRXKK (SEQ ID NO:161) in
30 which X is tryptophan and SEISY-EVEFRXKK (SEQ ID NO:162); KTEEISEISY-EVEFRXKK (SEQ ID NO:163); GLTNIKTEEISEISY-EVEFRXKK (SEQ ID NO:164);

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NO:164); TRPGSGLTNIKTEEISEISY-EVEFRXKK (SEQ ID NO:165); Biotin-SEISY-EVEFRXKK (SEQ ID NO:166); Biotin-KTEEISEISY-EVEFRXKK (SEQ ID NO:167); Biotin-GLTNIKTEEISEISY-EVEFRXKK (SEQ ID NO:168); Biotin-TRPGSGLTNIKTEEISEISY-EVEFRXKK (SEQ ID NO:169) in which X is oregon
5 green or other fluorescent moiety. Given this disclosure, it is contemplated that one of skill in the art may modify and extend any of the peptide substrates disclosed herein to provide additional useful Asp2 substrates.

Other preferred peptides of the present invention include SEISY-EVEFRWKK (SEQ ID NO:190), GLTNIKTEEISEISY-EVEFRWKK (SEQ ID
10 NO:191), Biotin-KEISEISY-EVEFR(Cys-Oregon Green)KK (SEQ ID NO:192) and Biotin -GLTNIKTEEISEISY-EVEFR(Cys-Oregon Green)KK (SEQ ID NO:193). In the latter two peptides the biotin facilitates capture with streptavidin and the Oregon Green provides fluorescent emission superior to the emission from the tryptophan in the peptides of SEQ ID NO: 190 and SEQ ID NO:191.

15 In the substrates SEISY-EVEFRWKK (SEQ ID NO:190) and GLTNIKTEEISEISY-EVEFRWKK (SEQ ID NO:191), the 10 N-terminal amino acids of peptide SEISY-EVEFRWKK (SEQ ID NO:190) are identical to those of peptide SEISY-EVEFR (SEQ ID NO:152). The difference between the two peptide is the presence of a tryptophan and two lysines in the peptide of SEQ ID NO:190.
20 SEISY-EVEFRWKK (SEQ ID NO:190) and SEISY-EVEFR (SEQ ID NO:152) have comparable activities towards Hu-Asp, but the addition of the fluorescent tryptophan increased the sensitivity of the HPLC assay. Moreover the addition of two lysines increased solubility. Hu-Asp activity was tested towards SEISY-EVEFRWKK (SEQ ID NO:190) and GLTNIKTEEISEISY-EVEFRWKK (SEQ ID NO:191). As expected
25 the peptide of SEQ ID NO:191 was much more active than the peptide of SEQ ID NO:190. The activity difference between the two peptides was particularly dramatic in the pH range 4.0-4.6. Specifically, it was 6.3-4.8 times higher for the peptide of SEQ ID NO:191. The activity of peptide of SEQ ID NO:190 decreased more rapidly than the activity of peptide of SEQ ID NO:191 with increasing ionic strength. Both
30 peptides were inhibited by increasing concentrations of DMSO. In studies conducted at 100 mM NaOAc/HOAc, pH 4.5, the peptide of SEQ ID NO:190 exhibited a $V_{\max}=185\text{nmol/mg/min}$ and a $K_m=86.7\mu\text{M}$, while the peptide of SEQ ID NO:191

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exhibited a $V_{\max}=1260\text{nmol/mg/min}$ and a $K_m=43.1\mu\text{M}$. As expected, the N-terminus elongation increases activity. Moreover, the internally quenched peptide substrate (MCA)Ac-SEVNL-DAEFRK(Dnp)RR-NH₂ (SEQ ID NO:195), that was made after the Swedish mutant APP encompassing the β -secretase cleavage site, exhibited a

5 $V_{\max}=18\text{nmol/mg/min}$ and a $K_m=7\mu\text{M}$. Remarkably, a 70-fold improvement in V_{\max} was achieved in going from the Swedish mutant peptide of SEQ ID NO:195 to the peptide of SEQ ID NO:191. The development of the substrates of SEQ ID NO:190 and SEQ ID NO:191 is of great value in Hu-Asp inhibition studies and in studies concerning recombinant Hu-Asp refolding from *E. coli* inclusion bodies.

10 The peptide substrates of the present invention may be any length of amino acids so long as the amino acids comprise a β -secretase cleavage site that is not depicted in any of the sequences of SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID

15 NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40. Preferably, the novel peptide substrates for Hu-Asp₂, are at least about five amino acids in length, in certain embodiments the novel peptides of the present invention may comprise a contiguous amino acid sequence of about 5; 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37,

20 38, 39, 40, 45, 50, or more amino acids.

In general, the peptides of the present invention may be defined by a basic motif which is in conformance with the Schechter and Berger nomenclature and comprises $P_2P_1-P_1'P_2'P_3'$; $P_2P_1-P_1'P_2'P_3'P_4'$; $P_3P_2P_1-P_1'P_2'P_3'$; $P_3P_2P_1-P_1'P_2'P_3'P_4'$; $P_4P_3P_2P_1-P_1'P_2'P_3'$; $P_4P_3P_2P_1-P_1'P_2'P_3'P_4'$ in which P_2 is a charged amino

25 acid, a polar amino acid, or an aliphatic amino acid but not an aromatic; P_1 is an aromatic amino acid or an aliphatic amino acid but not a polar amino acid or a charged amino acid; P_1' is a charged amino acid, an aliphatic amino acid or a polar amino acid, but not an aromatic amino acid; P_2' is an aliphatic polar amino acid or an aromatic amino acid but not a charged amino acid; P_3' is preferably a charged

30 amino acid but may be any other amino acid; P_3 is a polar amino acid, an aliphatic amino acid, or an aromatic amino acid but not a charged amino acid; P_4 is a charged

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amino acid, a polar amino acid or an aliphatic amino acid but not an aromatic amino acid and P_4' is preferably an aromatic amino acid but may be any other amino acid.

Ultimately, it is contemplated that the sequences of the novel substrates may comprise a sequence of $P_n \dots P_1 - P_1' \dots P_n'$. The length of the peptide may be of any length so long as the substrate comprises a β -secretase cleavage site that can be cleaved by a Hu-Asp2. The integer defined by n may be any integer so long as the substrate comprises a β -secretase cleavage site that can be cleaved by Hu-Asp2. Similarly, the integer defined by n' may be any integer so long as the substrate comprises a β -secretase cleavage site that can be cleaved by Hu-Asp2. It is contemplated that n and n' may be the same or different. Preferably, the β -secretase cleavage site is capable of being cleaved by a human aspartyl protease encoded by the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:3 (including the mature forms and biologically active fragments thereof).

The terms aromatic amino acid, charged amino acid, aliphatic amino acid and polar amino acid are well known to those of skill in the art and their usage in the present invention is consistent with the terms of the art. For example, the aromatic amino acids are phenylalanine, tyrosine and tryptophan, the charged amino acids (at physiological pH) are lysine, arginine, aspartate, glutamate and occasionally histidine and the polar amino acids are aspartic acid, glutamic acid, asparagine and glutamine, the amino acids presenting aliphatic side chains are glycine, alanine, valine, leucine, isoleucine, serine and threonine.

In considering the particular amino acid to be positioned at any of the position of $P_4, P_3, P_2, P_1, P_1', P_2', P_3'$ or P_4' it may be useful to consider the hydropathic index of amino acids at each of the positions in a peptide known to be an effective substrate for Hu-Asp2 as described herein and substitute a given amino acid with one of a similar hydropathic index. It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of a resultant protein or peptide, which in turn defines the interaction of that protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte & Doolittle, *J.*

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Mol. Biol., 157(1):105-132, 1982, incorporated herein by reference). Generally, amino acids may be substituted by other amino acids that have a similar hydropathic index or score and still result in a protein with similar biological activity *i.e.*, still obtain a biological functionally equivalent protein or peptide. In the context of the peptides of the present invention, a biologically functionally equivalent protein or peptide will be one which is still cleaved by β -secretase at a rate exceeding the rate of cleavage of a nature APP peptide comprising SEQ ID NO: 20.

In addition, the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As such, an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein.

Table 6 depicts exemplary amino acids that will be useful at each of positions P_4 , P_3 , P_2 , P_1 , P_1' , P_2' , P_3' and P_4'

	P_4	P_3	P_2	P_1	\downarrow	P_1'	P_2'	P_3'	P_4'
B_1	E	A	N	Y		E	V	E	F
B_2	G	V	L	L		A	A	G	W
B_3	I	I	K	M		D	N	F	G
B_4	D	S	S	Nle		M	T	H	A
B_5	T	H	G	F		Q	L	C*	H
B_6	C*	Y	T	H		S	F	S	P
B_7	S	T	D			G	S		G
B_8		F	A						N
B_9			Q						S
B_{10}			E						E

C* refers to Cysteic Acid

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It is envisioned that peptides may be constructed from the above table that have for example at position P_1 , any of the amino acids B_1 through B_7 in the P_1 column. Thus particular substrates that may be useful in the context of the present invention include

- 5 •ANY-EVEF (SEQ ID NO:49); E•NY-EVEF (SEQ ID NO: 50); EA•Y-EVEF (SEQ ID NO:51); EAN♦-EVEF (SEQ ID NO:52); EANY-◇VEF (SEQ ID NO:53); EANY-E△EF (SEQ ID NO: 54); EANY-EV▷F (SEQ ID NO: 55); EANY-EVE△ (SEQ ID NO: 56); •VLL-AAGW (SEQ ID NO: 57); G•LL-AAGW (SEQ ID NO: 58); GV•L-AAGW (SEQ ID NO: 59); GVL ♦-AAGW (SEQ ID NO: 60); GVL L-◇AGW (SEQ ID NO: 61); GVL L-A △GW (SEQ ID NO: 62); GVL L-AA ▷W (SEQ ID NO: 63); GVL L-AAG△ (SEQ ID NO: 64); •IKM-DNFG (SEQ ID NO: 65); I•KM-DNFG (SEQ ID NO: 66); II•M-DNFG (SEQ ID NO: 67); IIK♦-DNFG (SEQ ID NO: 68); IIKM-◇NFG (SEQ ID NO: 69); IIKM-D△FG (SEQ ID NO: 70); IIKM-DN▷G (SEQ ID NO: 71); IIKM-DNF△(SEQ ID NO: 72); •SSNIE-MTHA (SEQ ID NO: 73); D•SNIE-MTHA (SEQ ID NO: 74); DS•NIE-MTHA (SEQ ID NO: 75); DSS♦-MTHA (SEQ ID NO: 76); DSSNIE-◇THA (SEQ ID NO: 77); DSSNIE-M△HA (SEQ ID NO: 78); DSNIE-MT▷A (SEQ ID NO: 79); DSNIE-MTH△(SEQ ID NO: 80); •HGF-QLC*H (SEQ ID NO: 81); T•GF-QLC*H (SEQ ID NO: 82); TH•F-QLC*H (SEQ ID NO: 83); THG♦-QLC*H (SEQ ID NO: 84); THGF-◇LC*H (SEQ ID NO: 85); THGF-Q△C*H (SEQ ID NO: 86); THGF-QL▷H (SEQ ID NO: 87); THGF-QLC*△ (SEQ ID NO: 88); •YTH-SFSP (SEQ ID NO: 89); C*•TH-SFSP (SEQ ID NO: 90); C*Y•H-SFSP (SEQ ID NO: 91); C*YT♦-SFSP (SEQ ID NO: 92); C*YTH-◇FSP (SEQ ID NO: 93); C*YTH-S△SP (SEQ ID NO: 94); C*YTH-SF▷P (SEQ ID NO: 95); C*YTH-SFS△ (SEQ ID NO: 96); •TDX-GSXG (SEQ ID NO: 97); S•DX-GSXG (SEQ ID NO: 98); ST•X-GSXG (SEQ ID NO: 99); STD♦-GSXG (SEQ ID NO: 100); STDX-◇SXG (SEQ ID NO: 101); STDX-G△XG (SEQ ID NO: 102); STDX-GS▷G (SEQ ID NO: 103); STDX-GSX△(SEQ ID NO: 104); •FAX-XXXN (SEQ ID NO: 105); X•AX-XXXN (SEQ ID NO: 106); XF•X-XXXN (SEQ ID NO: 107); XFA♦-XXXN (SEQ ID NO: 108); XFAX-◇XXN (SEQ ID NO: 109); XFAX-X△XN (SEQ ID NO: 110); XFAX-XX▷N (SEQ ID NO: 111); XFAX-XXX△ (SEQ ID NO: 112); where "X"

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is any amino acid; "•" denotes for example, E, G, I, D, T, C* S; "▼" denotes for example, A, V, I, S, H, Y, T, F; "◄" denotes for example, N, L, K, S, G, T, D, A, Q, E; "◆" denotes for example, Y, L, M, Nle, F, H; "◇" denotes for example, E, A, D, M, Q, S, G; "△" denotes for example, V, A, N, T, L, F, S; "▷" denotes for example, E, G, F, H, C*, S and "Δ" denotes for example, F, W, G, A, H, P, G, N, S, E.

Particularly preferred peptides of the present invention include KVEANY-EVEGERKK (SEQ ID NO:5); KVEANY-EVEGERCKK (SEQ ID NO:6); KVEANY-AVEGERKK (SEQ ID NO:7); KVEANY-AVEGERCKK (SEQ ID NO:8); EANY-EVEF (SEQ ID NO:9); GVLL-AAGW (SEQ ID NO: 10); IIKM-DNFG (SEQ ID NO:11); DSSNle-MTHA (SEQ ID NO:12); THGF-QLC*H (SEQ ID NO:13); CYTH-SFSP (SEQ ID NO:14); STFX-GSXG (SEQ ID NO:15; XFAX-XXXN (SEQ ID NO: 16); XXQX-XXXS (SEQ ID NO:17); and XXEX-XXXE (SEQ ID NO:18).

The above teachings enable peptide substrates for Hu-Asp2 that will prove useful in the identification of modulators of Hu-Asp2 and/or modulators of Aβ plaque formation. Methods of making and using the above-identified substrates and variants thereof are described in greater detail herein below.

II. Derivatives of the Novel Hu-Asp2 Peptide Substrates

The Hu-Asp2 substrate peptide herein provide useful core structures to construct derivatives. Such derivatives may be fusion proteins or peptides that comprise the above discussed substrates as part of their sequence, or they may be labeled or otherwise modified Hu-Asp2 substrates in which the labeling or modification may be used to facilitate the purification of the peptide, detection of the peptide itself or a detection of the cleavage product of the peptide substrate upon the action of Hu-Asp. Exemplary modifications are described in further detail herein below.

A. Fusion Polypeptides

In addition to the novel peptide substrates described above, the

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present invention further contemplates the generation terminal additions, also called fusion proteins or fusion polypeptides, of the Hu-Asp2 substrates described above or identified according to the present invention. This fusion polypeptide generally has all or a substantial portion of the native molecule (*i.e.*, the Hu-Asp2 peptide substrates discussed above), linked at the N- and/or C-terminus, to all or a portion of a second or third polypeptide. It is contemplated that the fusion polypeptide may be produced by recombinant protein production or indeed by automated peptide synthesis as discussed elsewhere in the specification.

General principles for designing and making fusion proteins are well known to those of skill in the art. For example, fusions typically employ leader sequences from other species to permit the recombinant expression of a protein or peptide in a heterologous host. Another useful fusion includes the addition of an immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion polypeptide. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. The recombinant production of these fusions is described in further detail elsewhere in the specification. Other useful fusions include linking of functional domains, such as active sites from enzymes, glycosylation domains, cellular targeting signals or transmembrane regions.

More particularly, the present invention contemplates a fusion polypeptide in which there is a first component comprising the Hu-Asp2 cleavage site containing Hu-Asp2 peptide substrates of the present invention attached to a second component comprising a transmembrane domain. In additional embodiments, the fusion polypeptide further may comprise a third component which comprises a reporter gene product. In still further embodiments, the fusion polypeptides may further comprise a tagged sequence component. A particularly preferred fusion polypeptide is one which comprises a reporter gene product on one side of an intracellular transmembrane domain sequence, a short stretch of sequence containing the Hu-Asp2 cleavage site attached to a transmembrane domain with an ER/Golgi target signal, and tagged sequence on the other side of the transmembrane domain.

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It is contemplated that the distance between the cleavage site and the start of a transmembrane domain is about 20-40 amino acids in order to mimic the steric properties of the APP β secretase cleavage domain. This distance may be generated from the protein conferring the transmembrane region or it may be created by means of a heterologous peptide linker. Preferably, this region is from the transmembrane protein. The transmembrane domain component of the fusion polypeptide may be essentially any transmembrane domain component that will target and locate to the Golgi or endoplasmic reticulum of a given cell. Particularly preferred membrane targeting sequences include but are not limited to the transmembrane domain of galactosyltransferase (see for example Genbank accession number AF155582), the transmembrane domain of sialyl transferase (see for example Genbank accession number NM_003032); the transmembrane domain of human aspartyl protease 1 (Asp1; see for example Genbank accession number AF200342); the transmembrane domain of human aspartyl protease 2 (Asp2; see for example Genbank accession number NM_012104); the transmembrane domain of syntaxin 6 (see for example Genbank accession number NM_005819); the transmembrane domain of acetylglucosaminyl transferase (see for example Genbank accession number NM_002406) and the transmembrane domain of APP (see for example Genbank accession number A33292). The Genbank accession numbers given above detail the complete protein sequence. For the purposes of the present invention all or part of the transmembrane domains of these proteins may be used. In specific embodiments, residues 454-477 of the Asp2, residues 598-661 of APP (*e.g.*, of APP 695), residues 4-27 of galactosyltransferase, residues 470-492 of Asp1, residues 10-33 of sialyltransferase, residues 7-29 of acetylglucosaminyl transferase and residues 261-298 of syntaxin 6 will be particularly useful in this regard.

The reporter gene product used in the fusion polypeptides of the present invention may be any reporter protein commonly used by those of skill in the art. Exemplary reporter proteins include but are not limited to luciferase; secreted alkaline phosphatase (SEAP); β -galactosidase; β -glucuronidase; green fluorescent protein and chloramphenicol acetyl transferase.

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Other particular embodiments further contemplate a tagged sequence as a fourth component of the fusion polypeptides of the present invention. There are various commercially available fusion protein expression systems that may be used to provide a tagged sequence in this context of the present invention. Particularly useful systems include but are not limited to the glutathione S-transferase (GST) system (Pharmacia, Piscataway, NJ), the maltose binding protein system (NEB, Beverley, MA), the FLAG system (IBI, New Haven, CT), and the 6xHis system (Qiagen, Chatsworth, CA). These systems are capable of producing recombinant polypeptides bearing only a small number of additional amino acids, which are unlikely to affect the biologically relevant activity of the recombinant fusion protein. For example, both the FLAG system and the 6xHis system add only short sequences, both of which are known to be poorly antigenic and which do not adversely affect folding of the polypeptide to its native conformation. Another N-terminal fusion that is contemplated to be useful is the fusion of a Met-Lys dipeptide at the N-terminal region of the protein or peptides. Such a fusion may produce beneficial increases in protein expression and/or activity. Specific tagged sequences that are contemplated for use in the present invention include the FLAG tag sequence DYKDDDDK (SEQ ID NO: 186) and residues 662-695 of APP.

A typical example of a preferred fusion protein of the present invention is one in which SEAP is fused to either partial or full-length insulin B chain which comprises an Asp2 cleavage site containing one of the novel Hu-Asp2 substrates described herein and the transmembrane domain of Hu-Asp2 (residue 454-477) together with a short C-terminal Flag-tagged tail. The sequence of an exemplary fusion polypeptide is depicted in the FIG. 1 (SEQ ID NO:128). In order to monitor cleavage of the chimeric construct by Hu-Asp2, the chimeric construct used alkaline phosphatase as a reporter protein. Since the peptides that can be cleaved in an *in vitro* assay may not be aligned well with the protease in a cellular condition, the sequences inserted between SEAP and transmembrane domain may subject to change to obtain optimal condition. Such optimization may employ peptide linkers that will provide the optimum distance between the reporter protein and the cleavage site. The Hu-Asp2 transmembrane domain will ensure efficient

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delivery of the insulin or ubiquitin based substrate to the cellular environment where it can bind active Hu-Asp2 and be cleaved.

5 An alternative design uses the C-terminal 97 amino acids from APP to mimic its natural spacing and membrane domain. Essentially, this construct will have APP transmembrane domain and most of A β peptide sequences except that the cleavage site is mutated from KM-DAE (SEQ ID NO:129) to a sequence of P₂P₁-P₁'P₂'P₃' as defined above.

10 When the fusion protein co-expresses with Hu-Asp2 in a cell line or expresses in a cell line that produces higher Hu-Asp2 activity, Hu-Asp2 will cleave the fusion polypeptide to release SEAP into the cell medium. Thus, the protease activity may be monitored based on the SEAP activity in the medium.

As discussed in section I above, SEVSY- \downarrow -EAEFR (SEQ ID NO:144) and SEVSY- \downarrow -EVEFR (SEQ ID NO:141) are exemplary Asp2 peptide substrates of the present invention based on mutations of the native structure of APP. The former peptide has activity comparable to the Swedish mutation, and the latter peptide has activity superior to the Swedish mutation. In the case of SEVSY- \downarrow -EVEFR (SEQ ID NO:141) the amino acids surrounding the β -secretase cleavage site in APP have been changed from ...KM/NL- \downarrow -DA... (in the wild type SEQ ID NO:139/Swedish mutation SEQ ID NO:142) to ...SY- \downarrow -EV.... (SEQ ID NO:140)

20 The experimental data herein indicates that the above-indicated change of only four amino acids in the APP sequence will produce an excellent APP-modified substrate for Asp2 that will give easily measurable levels of A β (or other detectable cleavage products) in cell-based assays. Thus, it is envisioned that the sequences SY- \downarrow -EA (SEQ ID NO:187), or SY- \downarrow -EV (SEQ ID NO:140), or SY- \downarrow -AV (SEQ ID NO:188), or analogues thereof, be fused to sequences that have the ability to anchor the fusion proteins to a desired intracellular membrane such as the ER or Golgi compartments. The sequences SY- \downarrow -EA (SEQ ID NO:187), SY- \downarrow -EV (SEQ ID NO:140), or SY- \downarrow -AV (SEQ ID NO:188) are used to replace the sequence KM- \downarrow -DA (SEQ ID NO:139) in the wild type APP according to the scheme of FIG. 2

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30 under the column labeled as "1". The peptide data herein indicates that these constructs will be readily cleaved by β -secretase, relative to cleavage rates of native

APP.

FIG. 2 also describes in general terms, chimeric substrates for Asp2 cell based assays of the invention in which a reporter gene product such as SEAP is used on the ectodomain of an intracellular transmembrane segment. In a preferred embodiment, such a chimeric protein includes a short stretch of sequence containing the Asp2 cleavage site of the peptides of the present invention attached to a transmembrane domain with an ER/Golgi target signal in the middle, and a tagged sequence on the opposing side of the membrane.

An exemplary construct may be obtained by fusing SEAP to APP-CT99. The junction between SEAP and APP-CT99 is modified as shown in FIG. 2 under "2" to enhance cleavage by Asp2. Another construct may be based upon a cleavage site in ubiquitin (FIG. 2, column "3"). Specifically, SEAP is fused to ubiquitin which is C-terminally extended with the transmembrane domain of Asp2 (residue 454-477), and a short C-terminal Flag-tail. In this construct, the cleavable sequence of ubiquitin KTITL↓-EVEPS (SEQ ID NO:120) is modified to enhance cleavage. For example, T at P₂ is substituted by N or S, and L at P₁ is substituted by Y. Other possible substitutions are indicated in FIG. 2 next to each column, and in the experimental details described herein.

When ubiquitin is used as a fusion protein, ubiquitin C-terminal residues Gly-Gly are preferably changed to some other amino acids to avoid cleavage after ubiquitin C-terminus by ubiquitin hydrolases. The Asp2 transmembrane domain will ensure efficient delivery of the construct of interest to the cellular environment where it can be recognized by active Asp2, and cleaved. When the latter fusion protein is co-expressed with Asp2 in a cell line or expressed in a cell line that produces higher Asp2 activity, Asp2 will recognize and cleave the substrate. This event will release the polypeptide (located N-terminally to the cleavage site) into the cell medium, thus allowing direct assay of the protease activity.

B. Other Modifications

In addition to providing fusion polypeptides as already described, the

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invention provides fusion proteins or peptide substrates that are further modified to incorporate, for example, a label or other detectable moiety.

Preferred peptide substrates will comprise internally quenched labels that result in increased detectability after cleavage of the peptide substrates. The peptide substrates may be modified to have attached a paired fluorphore and quencher including but not limited to 7-amino-4-methyl coumarin and 2,4-dinitrophenol, respectively, such that cleavage of the peptide by the Hu-Asp results in increased fluorescence due to physical separation of the fluorphore and quencher, which are attached on opposite sides of the scissile bond. Other paired fluorphores and quenchers include bodipy-tetramethylrhodamine and QSY-5 (Molecular Probes, Inc.). In a variant of this assay, biotin or another suitable tag may be placed on one end of the peptide to anchor the peptide to a substrate assay plate and a fluorphore may be placed at the other end of the peptide. Useful fluorphores include those listed above as well as Europium labels such as W8044 (EG&g Wallac, Inc.). A preferred label is oregon green that may be attached to a Cys residue. Cleavage of the peptide by Asp2 will release the fluorphore or other tag from the plate, allowing compounds to be assayed for inhibition of Asp2 proteolytic cleavage as shown by an increase in retained fluorescence. Preferred colorimetric assays of Hu-Asp proteolytic activity utilize other suitable substrates that include the P₂ and P₁ amino acids comprising the recognition site for cleavage linked to o-nitrophenol through an amide linkage, such that cleavage by the Hu-Asp results in an increase in optical density after altering the assay buffer to alkaline pH.

Further, the peptides may be labeled using labels well known to those of skill in the art, *e.g.*, biotin labels are particularly contemplated. The use of such labels is well known to those of skill in the art and is described in, *e.g.*, U.S. No. Patent 3,817,837; U.S. Patent No. 3,850,752; U.S. Patent No. 3,996,345 and U.S. Patent No. 4,277,437. Other labels that will be useful include but are not limited to radioactive labels, fluorescent labels and chemiluminescent labels. U.S. Patents concerning use of such labels include for example U.S. Patent No. 3,817,837; U.S. Patent No. 3,850,752; U.S. Patent No. 3,939,350 and U.S. Patent No. 3,996,345. Any of the peptides of the present invention may comprise one, two, or more of any

of these labels.

Other derivatives specifically contemplated by the present invention include non-hydrolyzable derivatives such as statine derivatives of the β -secretase substrates of the present invention. Statine-containing peptides are recognized as having inhibitory effects on aspartyl proteases (Shewale, J. G.; Takahashi, R.; Tang, J., *Aspartic Proteinases and Their Inhibitors*, Kostka, V., Ed. Walter de Gruyter: Berlin (1986) pp. 101-116). Examples of statine inhibitors of cathepsin D (Lin, T.-Y.; Williams, J. *Biol. Chem.* (1979), 254, 11875-11883; Rich and Agarwal, N. S., *J. Med. Chem.* (1986) 29 (2519-2524)), and for plasmepsin (Silva, A. M. et al., *Proc. Natl Acad Sci*, 1996, 93, 10034-10039) also have been described.

Statine is a non-standard amino acid residue present in pepstatin and is known by the chemical name (3S, 4S)-4-amino-3 hydroxy-6 methylheptanoic acid and is further identified in the Merck index (11th Ed.) At monograph 8759. Statine is readily commercially available, for example from Sigma-Aldrich (St Louis, MO). The three letter abbreviation for statine is Sta in the peptide art. Statine derivatives may be prepared by methods disclosed in U.S. Patent 4,397,786. Other methods are described in *The Peptides*, Vol. 5: *Analysis, Biology*; (Academic Press, NY; 1983); Kessler and Schudok, *Synthesis* (6) 457-8 (1990); Nishi and Morisawa, *Heterocycles* 29(9), 1835-42 (1989), each incorporated herein by reference. Thus, those of skill in the art will be able to use such techniques to produce statine derivatives of the present invention. WO 00/77030 describes statine-derived peptide inhibitors of the β -secretase enzyme and is specifically incorporated herein by reference as teaching methods of producing statine derivatives of peptides.

In addition to statine derivatives, many other non-hydrolyzable peptide bonds are known in the art, along with procedures for synthesis of peptides containing such bonds. Non-hydrolyzable bonds include --[CH₂NH]-- reduced amide peptide bonds, --[COCH₂]-- ketomethylene peptide bonds, --[CH(CN)NH]-- (cyanomethylene)amino peptide bonds, --[CH₂CH(OH)]-- hydroxyethylene peptide bonds, --[CH₂O]-- peptide bonds, and --[CH₂S]-- thiomethylene peptide bonds (see e.g., U.S. Patent 6,172,043).

III. Aspartyl Protease.

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In addition to novel peptide substrates, the present invention is directed to methods of using such peptide substrates in various Hu-Asp2 assays. The present section provides a discussion of these proteins that have a β -secretase activity.

5 PCT Publication number WO 00/17369, specifically incorporated herein by reference in its entirety, describes methods and compositions relating to the identification and characterization of the β secretase enzyme, termed herein throughout as Asp2. In addition, U.S. Patent Application Serial No. 09/416,901 filed October 13, 1999, also is incorporated herein by reference in its entirety as
10 providing additional disclosure regarding the nature, function, and characterization of Asp2 and the role these proteases have in AD.

These patent applications describe two isoforms of Human Asp2 referred to as Hu-Asp-2(a) and Hu-Asp2(b), may be employed in the context of the present invention, as well as fragments, analogs, orthologs, and variants thereof.
15 All such forms may be employed in the context of the present invention, with forms that are most similar to native forms in Alzheimer's patients being preferred.

In particular embodiments, the apartyl proteases useful in the present invention are encoded by SEQ ID NO:1 or SEQ ID NO:3. The nucleic acid of SEQ ID NO:1 is predicted to encode a sequence of SEQ ID NO: 2 which is the human
20 Asp-2(a), predicted amino acid sequence. The Asp2(a) amino acid sequence includes a putative signal peptide comprising residues 1 to 21; and a putative pre-propeptide after the signal peptide that extends through residue 45 (as assessed by processing observed of recombinant Asp2(a) in CHO cells), and a putative propeptide that may extend to at least about residue 57, based on the observation of
25 an observed GRRIGS sequence which has characteristics of a protease recognition sequence. The Asp2(a) further includes a transmembrane domain comprising residues 455-477, a cytoplasmic domain comprising residues 478-501, and a putative alpha-helical spacer region, comprising residues 420-454, believed to be required for proper folding of Asp2, between the protease catalytic domain and the
30 transmembrane domain. The nucleic acid sequence of SEQ ID NO:3 is predicted to encode a sequence of SEQ ID NO: 4 which is the Human Asp-2(b), predicted amino

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acid sequence. The Asp2(b) amino acid sequence includes a putative signal peptide, pre-propeptide, and propeptide as described above for Asp2(a). The Asp2(b) further includes a transmembrane domain comprising residues 430-452, a cytoplasmic domain comprising residues 453-476, and a putative alpha-helical spacer region, comprising residues 395-429, believed to be required for proper folding of Asp2, between the protease catalytic domain and the transmembrane domain. As used herein, all references to "Hu-Asp2" should be understood to refer to both Hu-Asp2(a) and Hu-Asp2(b). The invention also contemplates the use of isolated or mutant Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides, as well as fragments thereof which exhibit aspartyl protease activity.

In an exemplary assay to determine the cleavage of the substrates of the present invention, Asp2 was prepared from High Five™ insect cells. Generally, the assays are run in solution at acidic pH (4.0 to 5.0), at 25-37°C in the presence of nM or sub-nanomolar enzyme. After a suitable period of incubation the reaction is stopped at a desired time by addition of 4% trifluoroacetic acid (TFA). Substrates and products are separated using reverse phase HPLC. The disappearance of the substrates and the appearance of the products are monitored by continuous measurement of the absorbance of the effluent at 220nm. Furthermore, if the peptide comprises a tryptophan residue, the monitoring may additionally or alternatively involve continuous monitoring of fluorescence of tryptophan fluorescence (excitation at 280 nm, emission at 248 nm) of the effluent. Peptides of the present invention which comprise an Oregon Green label either alone or in addition to biotin label are particularly suitable for high throughput screening. To this end, the peptides Biotin-KEISEISY-EVEFR(Cys-Oregon Green)KK (SEQ ID NO:192) and Biotin - GLTNIKTEEISEISY-EVEFR(Cys-Oregon Green)KK (SEQ ID NO:193) are particularly suited to high throughput screening assays.

In a typical assay, 210 nM enzyme and 200 μM substrate were incubated in 0.2 M sodium acetate at pH 4.5 in 100 μl volume at 37°C for 1 to 3 hours. The assay was stopped by the addition of 50 μl 4% TFA to lower the pH below the active range of the enzyme. Subsequently, 100 μl of this mixture was injected into a Hewlett Packard Model 1090 HPLC equipped with a Vydac column

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(4.6mm i.d. x 150 mm, 5 μ m) pre-equilibrated with 95% A (0.15% TFA in water), 5% B (0.15% TFA in acetonitrile). The constituents were then eluted from the column with the following linear gradients: 0-15 min: 5-50% B; 15-17 min: 50-100%B; 17-20 min: 100-5%B; 20-22 min: 5%B). The cleavage products were then
5 quantitated using an enhanced integrator.

In a variation of the above assay for substrates of SEQ ID NO:190 or its N-terminally extended version depicted in SEQ ID NO:191), the substrates are incubated with the enzyme to a final reaction solution volume of 200 μ l (although reaction mixtures of 50-100 μ l also were used) composed of 5-50 μ M peptide
10 substrate, 1-10 nM enzyme, 0.1 M sodium acetate buffer at pH 4 to 5. The reaction is allowed to proceed at 37 °C for 1 to 3 hrs and is terminated by addition of 100 μ l 4% TFA. Subsequently, an aliquot *e.g.*, 50 μ l to 100 μ l of the mixture are injected into an HPLC apparatus (Agilent 1100 series) equipped with an Alltech C₁₈ 3 μ M Rocket column (53mm length, 7mm ID, part No. 50605). Solvent A for the HPLC elution of
15 the substrates from this column was 0.1% TFA in water; Solvent B was 0.1% TFA in acetonitrile. The substrates were then eluted from the column with the following linear gradients: Time 0: 12% Solvent B; Time 4 30% Solvent B; Time 6 50% Solvent B; Time 6.5 90% Solvent B; Time 7 12% Solvent B; Time 8 12% Solvent B.

20 In addition to Hu-Asp, it is contemplated that the substrates of the present invention also may prove useful in assays employing other aspartyl proteases such as, for example, Asp1, Pepsin, gastricsin, cathepsin D, cathepsin E and Renin.

IV. Protein or Peptide Production and Purification

25 The present invention provides proteins and peptide for use in the identification of modulators of Hu-Asp2. Such proteins or peptides may be produced by conventional automated peptide synthesis methods or by recombinant expression.

30 A. Synthetic Peptide Production

The peptides or indeed even the full length fusion polypeptides of the

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invention can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, *Solid Phase Peptide Synthesis*, 2d. ed., Pierce Chemical Co., (1984); Tam *et al.*, *J. Am. Chem. Soc.*, 105:6442, (1983); Merrifield, *Science*, 232: 341-347, (1986); and Barany and Merrifield, *The Peptides*, Gross and Meienhofer, eds, Academic Press, New York, 1-284, (1979), each incorporated herein by reference. The novel Asp2 substrates of the invention which comprise the β -secretase cleavage site, can be readily synthesized and then screened in Hu-Asp2 screening assays.

In particularly preferred methods, the peptides of the present invention were synthesized by solid-phase technology employing a Model 433A from Applied Biosystems Inc. The purity of any given peptide substrate, generated through automated peptide synthesis or through recombinant methods may be determined using reverse phase HPLC analysis. Chemical authenticity of each peptide may be established by any method well known to those of skill in the art. In preferred embodiments, the authenticity is established by mass spectrometry as described in the examples.

Additionally, the peptides may be quantitated using amino acid analysis in which microwave hydrolyses are conducted. Such analyses may use a microwave oven such as the CEM Corporation's MDS 2000 microwave oven. The peptide (approximately 2 μ g protein) is contacted with 6 N HCl (Pierce Constant Boiling e.g., about 4 ml) with approximately 0.5% (volume to volume) phenol (Mallinckrodt). Prior to the hydrolysis, the samples are alternately evacuated and flushed with N_2 . The protein hydrolysis is conducted using a two-stage process. During the first stage, the peptides are subjected to a reaction temperature of about 100 °C and held at that temperature for 1 minute. Immediately after this step, the temperature is increased to 150 °C and held at that temperature for about 25 minutes. After cooling, the samples are dried and amino acid from the hydrolysed peptides samples are derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate to yield stable ureas that fluoresce at 395 nm (Waters AccQ•Tag Chemistry Package).

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The samples may be analyzed by reverse phase HPLC and quantification may be achieved using an enhanced integrator.

B. Recombinant Protein Production.

5 As an alternative to automated peptide synthesis, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression as described herein below. Recombinant methods are especially
10 preferred for producing longer polypeptides that comprise peptide sequences of the invention.

 From the disclosure of novel HuAsp2 substrate peptides sequences, it is possible to produce the peptides and fusion polypeptides by recombinant techniques. A variety of expression vector/host systems may be utilized to contain
15 and express the peptide or fusion polypeptide coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (*e.g.*, baculovirus); plant cell systems transfected with virus expression vectors
20 (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmid); or animal cell systems. Mammalian cells that are useful in recombinant protein productions include but are not limited to VERO cells, HeLa cells, Chinese hamster ovary (CHO) cell lines, COS cells (such as COS-7), W138, BHK, HepG2, 3T3,
25 RIN, MDCK, A549, PC12, K562 and 293 cells. Exemplary protocols for the recombinant expression of the peptide substrates or fusion polypeptides in bacteria, yeast and other invertebrates are described herein below.

 Expression vectors for use in prokaryotic hosts generally comprise one or more phenotypic selectable marker genes. Such genes generally encode,
30 *e.g.*, a protein that confers antibiotic resistance or that supplies an auxotrophic requirement. A wide variety of such vectors are readily available from commercial

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sources. Examples include pSPORT vectors, pGEM vectors (Promega), pPROEX vectors (LTI, Bethesda, MD), Bluescript vectors (Stratagene), pET vectors (Novagen) and pQE vectors (Qiagen). The DNA sequence encoding the given peptide substrate or fusion polypeptide is amplified by PCR and cloned into such a vector, for example, pGEX-3X (Pharmacia, Piscataway, NJ) designed to produce a fusion protein comprising glutathione-S-transferase (GST), encoded by the vector, and a protein encoded by a DNA fragment inserted into the vector's cloning site. The primers for the PCR may be generated to include for example, an appropriate cleavage site. Treatment of the recombinant fusion protein with thrombin or factor Xa (Pharmacia, Piscataway, NJ) is expected to cleave the fusion protein, releasing the substrate or substrate containing polypeptide from the GST portion. The pGEX-3X/HuAsp2 peptide construct is transformed into *E. coli* XL-1 Blue cells (Stratagene, La Jolla CA), and individual transformants were isolated and grown. Plasmid DNA from individual transformants is purified and partially sequenced using an automated sequencer to confirm the presence of the desired peptide or polypeptide encoding nucleic acid insert in the proper orientation.

While certain embodiments of the present invention contemplate producing the peptides or polypeptides using synthetic peptide synthesizers and subsequent FPLC analysis and appropriate refolding of the cysteine double bonds, it is contemplated that recombinant protein production also may be used to produce the peptide compositions. For example, induction of the GST/substrate fusion protein is achieved by growing the transformed XL-1 Blue culture at 37°C in LB medium (supplemented with carbenicillin) to an optical density at wavelength 600 nm of 0.4, followed by further incubation for 4 hours in the presence of 0.5 mM Isopropyl β -D-Thiogalactopyranoside (Sigma Chemical Co., St. Louis MO).

The GST fusion protein, expected to be produced as an insoluble inclusion body in the bacteria, may be purified as follows. Cells are harvested by centrifugation; washed in 0.15 M NaCl, 10 mM Tris, pH 8, 1 mM EDTA; and treated with 0.1 mg/ml lysozyme (Sigma Chemical Co.) for 15 minutes at room temperature. The lysate is cleared by sonication, and cell debris is pelleted by centrifugation for 10 minutes at 12,000 X g. The fusion protein-containing pellet is

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resuspended in 50 mM Tris, pH 8, and 10 mM EDTA, layered over 50% glycerol, and centrifuged for 30 min. at 6000 X g. The pellet is resuspended in standard phosphate buffered saline solution (PBS) free of Mg^{++} and Ca^{++} . The fusion protein is further purified by fractionating the resuspended pellet in a denaturing SDS polyacrylamide gel (Sambrook *et al.*, *supra*). The gel is soaked in 0.4 M KCl to visualize the protein, which is excised and electroeluted in gel-running buffer lacking SDS. If the GST/HuAsp2 fusion protein is produced in bacteria as a soluble protein, it may be purified using the GST Purification Module (Pharmacia Biotech).

The fusion protein may be subjected to thrombin digestion to cleave the GST from the mature HuAsp2 substrate peptide or the HuAsp substrate-containing polypeptide. The digestion reaction (20-40 μ g fusion protein, 20-30 units human thrombin (4000 U/mg (Sigma) in 0.5 ml PBS) is incubated 16-48 hrs. at room temperature and loaded on a denaturing SDS-PAGE gel to fractionate the reaction products. The gel is soaked in 0.4 M KCl to visualize the protein bands. The identity of the protein band corresponding to the expected molecular weight of HuAsp2 substrate or fusion polypeptide may be confirmed by partial amino acid sequence analysis using an automated sequencer (Applied Biosystems Model 473A, Foster City, CA).

Alternatively, the DNA sequence encoding the predicted substrate containing fusion polypeptide may be cloned into a plasmid containing a desired promoter and, optionally, a leader sequence (*see, e.g.*, Better *et al.*, *Science*, 240:1041-43, 1988). The sequence of this construct may be confirmed by automated sequencing. The plasmid is then transformed into *E. coli* using standard procedures employing $CaCl_2$ incubation and heat shock treatment of the bacteria (Sambrook *et al.*, *supra*). The transformed bacteria are grown in LB medium supplemented with carbenicillin, and production of the expressed protein is induced by growth in a suitable medium. If present, the leader sequence will effect secretion of the mature HuAsp2 substrate or fusion protein and be cleaved during secretion.

The secreted recombinant protein is purified from the bacterial culture media by the method described herein throughout.

Similarly, yeast host cells from genera including *Saccharomyces*,

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Pichia, and *Kluveromyces* may be employed to generate the recombinant peptide. Preferred yeast hosts are *S. cerevisiae* and *P. pastoris*. Yeast vectors will often contain an origin of replication sequence from a 2T yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Vectors replicable in both yeast and *E. coli* (termed shuttle vectors) may also be used. In addition to the above-mentioned features of yeast vectors, a shuttle vector will also include sequences for replication and selection in *E. coli*. Direct secretion of polypeptides expressed in yeast hosts may be accomplished by the inclusion of nucleotide sequence encoding the yeast I-factor leader sequence at the 5' end of the substrate-encoding nucleotide sequence.

Generally, a given substrate may be recombinantly expressed in yeast using a commercially available expression system, *e.g.*, the *Pichia* Expression System (Invitrogen, San Diego, CA), following the manufacturer's instructions. This system also relies on the pre-pro-alpha sequence to direct secretion, but transcription of the insert is driven by the alcohol oxidase (AOX1) promoter upon induction by methanol.

The secreted recombinant substrate is purified from the yeast growth medium by, *e.g.*, the methods used to purify substrate from bacterial and mammalian cell supernatants.

Alternatively, a synthetic DNA encoding the novel substrate of the invention may be cloned into the baculovirus expression vector pVL1393 (PharMingen, San Diego, CA; Luckow and Summers, *Bio/Technology* 6:47 (1988)). This substrate-containing vector is then used according to the manufacturer's directions (PharMingen) to infect *Spodoptera frugiperda* cells in sf9 protein-free media and to produce recombinant protein. The protein or peptide is purified and concentrated from the media using a heparin-Sepharose column (Pharmacia, Piscataway, NJ) and sequential molecular sizing columns (Amicon, Beverly, MA), and resuspended in PBS. SDS-PAGE analysis shows a single band and confirms the size of the protein, and Edman sequencing on a Porton 2090 Peptide Sequencer confirms its N-terminal sequence.

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Alternatively, the HuAsp2 substrate may be expressed in an insect system. Insect systems for protein expression are well known to those of skill in the art. In one such system, *Autographa californica nuclear polyhedrosis virus* (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. The substrate coding sequence is cloned into a nonessential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of substrate will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein coat. The recombinant viruses are then used to infect *S. frugiperda* cells or *Trichoplusia* larvae in which the substrate is expressed (Smith *et al.*, *J Virol* 46: 584, 1983; Engelhard EK *et al.*, *Proc Nat Acad Sci* 91: 3224-7, 1994).

Mammalian host systems for the expression of recombinant proteins also are well known to those of skill in the art. Host cell strains may be chosen for a particular ability to process the expressed protein or produce certain post-translation modifications that will be useful in providing protein activity. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be important for correct insertion, folding and/or function. Different host cells such as CHO, HeLa, MDCK, 293, WI38, and the like have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the introduced, foreign protein.

It is preferable that the transformed cells are used for long-term, high-yield protein production and as such stable expression is desirable. Once such cells are transformed with vectors that contain selectable markers along with the desired expression cassette, the cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The selectable marker is designed to confer resistance to selection and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clumps of stably transformed cells can be proliferated using tissue culture techniques

appropriate to the cell.

A number of selection systems may be used to recover the cells that have been transformed for recombinant protein production. Such selection systems include, but are not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in tk-, hgp_{rt}- or ap_{rt}- cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for dhfr, that confers resistance to methotrexate; gpt, that confers resistance to mycophenolic acid; neo, that confers resistance to the aminoglycoside G418; als which confers resistance to chlorsulfuron; and hygro, that confers resistance to hygromycin. Additional selectable genes that may be useful include trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine. Markers that give a visual indication for identification of transformants include anthocyanins, β -glucuronidase and its substrate, GUS, and luciferase and its substrate, luciferin.

C. Site-Specific Mutagenesis.

Site-specific mutagenesis is another technique useful in the preparation of individual Hu-Asp2 substrate peptide and more particularly fusion polypeptides that comprise as a component one of the Hu-Asp2 substrate peptides of the present invention. This technique employs specific mutagenesis of the underlying DNA (that encodes the amino acid sequence that is targeted for modification). The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

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The technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids also are routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a DNA sequence encoding the desired protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, taking into account the degree of mismatch when selecting hybridization (annealing) conditions, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

Of course, the above described approach for site-directed mutagenesis is not the only method of generating potentially useful mutant peptide species and as such is not meant to be limiting. The present invention also contemplates other methods of achieving mutagenesis such as for example, treating the recombinant vectors carrying the gene of interest mutagenic agents, such as hydroxylamine, to obtain sequence variants.

D. Protein Purification.

It will be desirable to purify the peptides of the present invention. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to

polypeptide and non-polypeptide fractions. Having separated the peptide or polypeptides of the invention from other proteins, the polypeptides or peptides of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to
5 homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography (FPLC) or even high performance liquid chromatography (HPLC).

10 Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded polypeptide, protein or peptide. The term "purified polypeptide, protein or peptide" as used herein, is intended to refer to a composition, isolated from other components, wherein the polypeptide, protein or peptide is purified to any degree relative to its
15 naturally-obtainable state. A purified polypeptide, protein or peptide therefore also refers to a polypeptide, protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a polypeptide, protein or peptide composition that has been subjected to fractionation to remove various other
20 components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the polypeptide, protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the
25 composition.

Various methods for quantifying the degree of purification of the polypeptide, protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by
30 SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of

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the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed polypeptide, protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified polypeptide, protein or peptide.

There is no general requirement that the polypeptide, protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, 76:425, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

V. Expression Constructs for use in the Production of the Substrates of the Invention.

In the present invention, it may be necessary to express the peptide substrates or the peptide-substrate fusion proteins of the present invention. To achieve such expression, the present invention will employ vectors comprising polynucleotide molecules for encoding the peptide substrates or the fusion proteins of the present invention, as well as host cell transformed with such vectors. Such polynucleotide molecules may be joined to a vector, which generally includes a selectable marker and an origin of replication, for propagation in a host. These elements of the expression constructs used in the present invention are described in further detail herein below.

The expression vectors include DNA encoding any of the given peptide or fusion polypeptide Hu-Asp2 substrates described above or below, operably linked to suitable transcriptional or translational regulatory sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, mRNA ribosomal binding sites, and appropriate sequences which control transcription and translation.

The terms "expression vector," "expression construct " or "expression cassette " are used interchangeably throughout this specification and are meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed.

The choice of a suitable expression vector for expression of the peptides or polypeptides of the invention will of course depend upon the specific host cell to be used, and is within the skill of the ordinary artisan. Examples of suitable expression vectors include pcDNA3 (Invitrogen) and pSVL (Pharmacia Biotech). A preferred vector for expression in the present invention is pcDNA3.1-Hygro (Invitrogen). Expression vectors for use in mammalian host cells may include transcriptional and translational control sequences derived from viral genomes. Commonly used promoter sequences and enhancer sequences which may

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be used in the present invention include, but are not limited to, those derived from human cytomegalovirus (CMV), Adenovirus 2, Polyoma virus, and Simian virus 40 (SV40). Methods for the construction of mammalian expression vectors are disclosed, for example, in Okayama and Berg (*Mol. Cell. Biol.* 3:280 (1983));
5 Cosman *et al.* (*Mol. Immunol.* 23:935 (1986)); Cosman *et al.* (*Nature* 312:768 (1984)); EP-A-0367566; and WO 91/18982.

The expression construct will comprise a nucleic acid region that encodes the particular peptide substrate or fusion polypeptide of the present invention. Coding regions for use in constructing such expression vectors should
10 encode at least the β -secretase cleavage of the peptides described herein although it is contemplated that larger polypeptides may be encoded as long as one the peptide generated comprises a β -secretase cleavage site that is amenable to cleavage by an aspartyl protease and preferably Hu-Asp.

In certain aspects of the present invention, the expression construct
15 may further comprise a selectable marker that allows for the detection of the expression of the peptide or polypeptide. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, neomycin, puromycin, hygromycin, DHFR, zeocin and histidinol. Alternatively, enzymes such as herpes simplex virus thymidine kinase (tk) (eukaryotic), β -
20 galactosidase, luciferase, or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed. Immunologic markers also can be employed. For example, epitope tags such as the FLAG system (IBI, New Haven, CT), HA and the 6xHis system (Qiagen, Chatsworth, CA) may be employed. Additionally, glutathione S-transferase (GST) system (Pharmacia, Piscataway, NJ), or the maltose binding
25 protein system (NEB, Beverley, MA) also may be used. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art. Particularly preferred selectable markers that may be employed in the present invention are neomycin
30 resistance or a GFP marker.

Expression requires that appropriate signals be provided in the

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vectors. The present section includes a discussion of various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that may be used to drive expression of the nucleic acids of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells also
5 are defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable cell clones expressing the products also are provided, as is an element that links expression of the drug selection markers to expression of the mutant phenotype.

In preferred embodiments, the nucleic acid encoding the given
10 peptide or the nucleic acid encoding a selectable marker is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene.

Nucleotide sequences are operably linked when the regulatory
15 sequence functionally relates to the DNA encoding the peptide substrate or the fusion polypeptide. Thus, a promoter nucleotide sequence is operably linked to a given DNA sequence if the promoter nucleotide sequence directs the transcription of the sequence. Similarly, the phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to
20 control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine
25 kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start
30 site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian

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terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, β -actin, rat insulin promoter, the phosphoglycerol kinase promoter and glyceraldehyde-3-phosphate dehydrogenase promoter, all of which are promoters well known and readily available to those of skill in the art, can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be

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optimized.

Selection of a promoter that is regulated in response to specific physiologic or synthetic signals can permit inducible expression of the gene product. Several inducible promoter systems are available for production of viral vectors.

5 One such system is the ecdysone system (Invitrogen, Carlsbad, CA), which is designed to allow regulated expression of a gene of interest in mammalian cells. It consists of a tightly regulated expression mechanism that allows virtually no basal level expression of the transgene, but over 200-fold inducibility.

Another useful inducible system is the Tet-Off™ or Tet-On™ system
10 (Clontech, Palo Alto, CA) originally developed by Gossen and Bujard (Gossen and Bujard, *Proc Natl Acad Sci U S A.* 15;89(12):5547-51, 1992; Gossen *et al.*, *Science*, 268(5218):1766-9, 1995).

In mammalian cells, the CMV immediate early promoter is often used to provide strong transcriptional activation. Modified versions of the CMV
15 promoter that are less potent have also been used when reduced levels of expression of the transgene are desired. Retroviral promoters such as the LTRs from MLV or MMTV are contemplated to be useful in the present invention. Other viral promoters that may be used include SV40, RSV LTR, HIV-1 and HIV-2 LTR, adenovirus promoters such as from the E1A, E2A, or MLP region, AAV LTR,
20 cauliflower mosaic virus, HSV-TK, and avian sarcoma virus.

In some embodiments, regulatable promoters may prove useful. Such promoters include for example, those that are hormone or cytokine regulatable. Hormone regulatable promoters include MMTV, MT-1, ecdysone and RuBisco as well as other hormone regulated promoters such as those responsive to thyroid,
25 pituitary and adrenal hormones.

Another regulatory element contemplated for use in the present invention is an enhancer. These are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many
30 individual elements, each of which binds to one or more transcriptional proteins. The basic distinction between enhancers and promoters is operational. An enhancer

region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization. Enhancers useful in the present invention are well known to those of skill in the art and will depend on the particular expression system being employed (Scharf D *et al* (1994) *Results Probl Cell Differ* 20: 125-62; Bittner *et al* (1987) *Methods in Enzymol* 153: 516-544).

Where an expression construct employs a cDNA insert, one will typically desire to include a polyadenylation signal sequence to effect proper polyadenylation of the gene transcript. Any polyadenylation signal sequence recognized by cells of the selected transgenic animal species is suitable for the practice of the invention, such as human or bovine growth hormone and SV40 polyadenylation signals.

Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences. The termination region which is employed primarily will be one selected for convenience, since termination regions for the applications such as those contemplated by the present invention appear to be relatively interchangeable. The termination region may be native with the transcriptional initiation, may be native to the DNA sequence of interest, or may be derived for another source.

In certain embodiments of the invention, the use of internal ribosome entry site (IRES) elements is contemplated to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, *Nature*, 334:320-325, 1988). IRES elements from two members of the picornavirus family (poliovirus and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988 *supra*), as well an IRES from a mammalian message (Macejak and Sarnow, *Nature*, 353:90-94, 1991). IRES elements can be linked to

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heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

VI. Use of the Substrates in Hu-Asp2 assays

In specific embodiments, the present invention involves assays to monitor the activity and/or function of Hu-Asp2 and more specifically, the β -secretase activity and/or function of Hu-Asp2. These assays will involve incubating in solution an Hu-Asp2 polypeptide with a suitable substrate of the present invention, using cleavage of the peptide substrate as a measure of Hu-Asp2 proteolytic activity.

A. Assay Formats

In specific embodiments, the invention relates to a method for the identification of agents that modulate the activity of human Asp2 aspartyl protease (Hu-Asp2). For example, in one variation, such method comprises steps of:

- (a) contacting any of the peptides or fusion proteins of the present invention and a composition comprising an Hu-Asp2 activity in the presence and absence of a test agent;
- (b) determining the cleavage of said peptide or fusion protein at the site between said P_1 and P_1' by said Hu-Asp2 in the presence and absence of the test agent; and
- (c) comparing said cleavage activity of the Hu-Asp2 in the presence of the test agent to the activity in the absence of the test agent

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to identify an agent that modulates said cleavage by the Hu-Asp2,

wherein an alteration in the Hu-Asp2 activity in the presence of the test agent identifies an agent that is a modulator of the Hu-Asp2 activity.

5 Agents that cause increased cleavage relative to the control (no test agent) are scored as agonists or stimulators of Asp2 proteolytic activity, whereas agents to cause decreased cleavage are scored as inhibitors. The inhibitors are of special interest because inhibitors of Asp2 activity have therapeutic and prophylactic indications for the treatment and prevention of Alzheimer's Disease or its
10 symptoms or progression.

Exemplary assays which can be modified by the use of peptides and fusion proteins of the invention (in place of APP and APP peptide sequences) are described in PCT Publication number WO 00/17369, and in U.S. Patent Application Serial No. 09/416,901, filed October 13, 1999, both incorporated herein by
15 reference for their teachings relating to Asp2 activity assays and assays for modulators of Asp2 activity. The Asp2 may be Hu-Asp2(a), Hu-Asp2(b), or biologically active fragments, analogs, or variants, thereof, for example. Non-human orthologs of human Asp2 also may be used in assays.

Such assays may be performed with Hu-Asp polypeptide in a cell free
20 system or with cultured cells that express Hu-Asp as well as variants or isoforms thereof. For example, in a cell-free system, the contacting step may be performed by mixing the Hu-Asp2 enzyme with the peptide or protein substrate of the invention, in the presence or absence of the test agent. For optimal results, the enzyme and the substrate preferably are substantially purified, mixed in defined and
25 controlled quantities, and mixed in appropriate buffers that optimize enzymatic activity and/or mimic physiological conditions. The determining step may involve a measurement of an N-terminal fragment, a C-terminal fragment, or both, or may involve measurement of another parameter indicative of cleavage. For example, the peptide substrate may contain a quenched label that becomes more detectable only
30 upon cleavage to separate the label from the quenching moiety. Alternatively, the peptide substrate may be fixed at the N-terminal or C-terminal end to a solid

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support. In this arrangement, cleavage may be measured by release from the solid support of a cleavage fragment. The release may be measured by increased label in the media, or decreased label attached to the solid support. Alternatively, the release may be measured by quantitative capture of the released peptide (*e.g.*, with an antibody).

In an exemplary capture assay, 384-well micro-titer plates are blocked with BSA enzyme (7.5nM) and 50μM of the compound to be tested are incubated for 1 hour and the reaction is initiated by the addition of 250nM substrate, for example Bition-KVEANYEVEGERC[cys-oregon green]KK. In the final assay conditions, the volume is 30μl/well (pH5.0); 50μM compound; 7.5nM Enzyme (or 15ng/well); 250nM substrate; 5 % DMSO and 0.001 % TWEEN-20. The assay is incubated overnight at room temperature and the reaction is terminated by the addition of Tris-HCl , pH 8.3. An aliquot containing 6.25 pmoles of substrate is removed and the cleaved and/or uncleaved biotinylated substrate is captured in a streptavidin coated plate. The plate is washed 3 time and buffer is added. The capture assay is monitored by reading the fluorescence emission of the oregon green on an LJL Analyst (Ex 485/Em 530).

Another assay that may be used herein is a fluorescent polarization assay. Fluorescence polarization is a sensitive, facile and non-destructive assay that can be exploited to monitor the effects of the β-secretase substrates of the present invention. It can be used to monitor the interaction of these substrates with the HuAsp2 enzyme. Under controlled conditions, fluorescence polarization measurements can reveal the extent of “molecular tumbling” of a fluorescent molecule in solution. For example, a small molecule with a compact molecular volume would be expected to tumble rapidly. If irradiated with polarized light the rapid movement of the molecule in solution would result in extensive depolarization of the light, and would yield a readout of “low” polarization value. Under the same conditions, the increased molecular volume of a large molecule or a large complex would slow the molecular rotation (tumbling) process. As a result, less polarization of the incident plane polarized light would result and a higher polarization value would be measured.

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By labeling a small ligand with a fluorescent probes, changes in the fluorescence polarization resulting from the interaction of the ligand with another system component can be measured. Such a method may be applied to measure the strength of interaction between an enzyme (HuAsp2) and a fluorescent enzyme substrate.

In an exemplary fluorescence polarization assay, in pre-blocked low affinity, black plates (384-wells) enzyme (0.5nM) and compound (10-20μM) are incubated for 30 minutes and the reaction initiated by the addition of 150nM substrate (*e.g.*, Biotin-GLNIKTEEISEISY-EVEFR[cys-oregon green]KK or a similar substrate containing a non-hydrolyzable bond at the P₁--P₁' position) to a final volume of 30μl/well. The final assay conditions are: 30μl/well volume at pH4.5; 50μM compound, 0.5nM enzyme or 1ng/well; 150nM substrate, 2% DMSA and 0.001% TWEEN-20. The assay is incubated for 3 hours at 37°C and the reaction terminated by the addition of a saturating concentration of immunopure streptavidin. The plate is then incubated at room temperature for 15 minutes and the fluorescent polarization measured on an L.J.L Acquest (Ex 485/Em 530).

Also contemplated by the present invention is a binding assay for detecting compounds that bind to the active site or at an allosteric site of the enzyme. For such determinations, the use of non-hydrolyzable derivatives of the substrates of the present invention is particularly preferred. In exemplary derivatives for such assays, the presence of a statine derivative at P₁ renders the peptides of the present invention non-hydrolyzable at the P₁--P₁' position. The substrates further may be modified with the addition of an appropriate fluorescent tag *e.g.*, BODIPY FL to facilitate detection.

In a specific example, a statine-containing peptide (SEVN[Sta]VAEFRGGC; SEQ ID NO:196) is synthesized and shown to inhibit HuAsp enzyme activity. A fluorescent derivative of this inhibitor, (SEVN[Sta]VAEFR(Bodipy FL)), also was synthesized and employed as described below. The fluorescence polarization of these statine-derived substrates in solution is minimal. However, upon interaction with HuAsp2 a dramatic increase in fluorescence polarization results. While the examples discussed in the present section refer to statine derivatives, it is contemplated that other non-hydrolyzable derivatives

containing a fluorescent moiety may be used in the fluorescence polarization assays.

A substrate of the present invention may be labeled as discussed above and used to develop a fluorescence polarization binding assay for the HuAsp2. The equilibrium dissociation constant (K_D) for the interaction between the enzyme and the substrate is determined by measuring fluorescence polarization changes which result from titrating the substrate with the enzyme.

To determine the K_D for the interaction of a substrate of the present invention with HuAsp2, various quantities of β -secretase may be combined with 3.1 nM fluorescent substrate and incubated at room temperature for 3 hours. Following the incubation, fluorescence polarization is determined using an LJL Analyst (96 well format) or a PanVera Beacon (single cuvette format). An exemplary assay is performed in 25 mM sodium acetate, 20% glycerol, pH 4.75. A graphic plot of the data obtained providing the polarization values on the vertical axis and the concentration of enzyme on the horizontal axis provides the binding isotherm for the determination of the K_D for the interaction of the enzyme with the substrate. The data may then be analyzed using the relation $P_x = P_F + (P_B - P_F) * [E] / (K_D + [E])$, where P=polarization value, x=sample, F=free inhibitor, B=bound inhibitor, E= β -secretase (Fluorescence Polarization Applications Guide, 1998; PanVera, Madison, WI) to obtain the K_D . This assay can be used to screen for compounds that bind to the active site of the enzyme.

Using the above assay, the fluorescence polarization binding assay may be validated and the K_D measured for a peptide substrate containing the wild-type or Swedish mutation. For example, such a initial assay may be performed using the fluorescent derivative, (SEVN[Sta]VAEFRC(Bodipy FL)). Subsequently, the assay may be modified for use in a competitive binding format for use in determining the activity of useful substrates of the present invention. A solution containing the SEVN[Sta]VAEFRC(Bodipy FL) substrate is titrated with a prospective competitive inhibitor, for example, 3.1nM SEVN[Sta]VAEFRC(Bodipy FL) substrate, 48nM enzyme and various concentrations of the prospective competitive inhibitor are incubated for an appropriate time to allow the reaction to progress. The data from this assay may be plotted on a graph where the vertical axis represents the polarization and the horizontal axis represents the competitive inhibitor concentration.

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Numerous cell-based embodiments also exist. For example, in one variation, the Hu-Asp2 is expressed in a cell, and the contacting comprises growing the cell in the presence of the peptide and in the presence and absence of the test agent. Cells which naturally express Asp2 may be selected. In a preferred
5 embodiment, a cell is recombinantly modified to express increased amounts of Asp2. Irrespective of which variation is used, the substrate peptide or fusion protein may be added to the cell system (*e.g.*, in the medium), or may be co-expressed by the cell along with the Asp2. For example, in a preferred
10 embodiment, the cell recombinantly expresses a fusion polypeptide described herein that includes a transmembrane domain that causes the peptide to localize to the ER or Golgi, and further includes appropriate tags, labels, fusion partners, reporter protein or the like as described herein to facilitate detection of cleavage.

In an exemplary cellular assay, a stable HEK-293 cell line expressing HA-gagged enzyme is transfected with APP variants. After transfection for 48 hours,
15 cell extracts are prepared for measuring Hu-Asp cleavage products C99 by Western blot with antibody C8. Equal amount of conditioned medium are used for measuring the levels of total secreted APP fragments with antibody 22C11 (Boehringer Mannheim) and secreted APP α , a fragment cleaved by α -secretase, with antibody 6E10 (Senetek, St. Louis, MO).

20 It will be appreciated that the activity measurements in the presence and absence of a test agent can be performed in parallel, or sequentially, in either order. Moreover, it may not be necessary to repeat the control measurements (*i.e.*, the measurements of cleavage in the absence of a test agent) in parallel with respect to every test agent, once a reliable baseline of enzymatic activity for particular
25 reaction conditions has been obtained. Gained knowledge of the enzymatic activity of Asp2 towards a particular substrate in the absence of inhibitors can be used as the basis for performing the comparison step.

Also, while the above discussion is generally made with reference to modulators of Hu-Asp2 activity, the assays of the invention also will identify
30 candidate substances that alter the production of Hu-Asp2, thereby increasing or decreasing the amount of Hu-Asp2 present as opposed to the per unit activity of the Hu-Asp2. Agents that decrease production of Hu-Asp2 also have indications for

treatment or prevention of Alzheimer's disease.

B. Candidate Substances.

As used herein the term "candidate substance" or "test substance" refers to any molecule that is capable of modulating Hu-Asp2 activity, and preferably Hu-Asp2 β -secretase activity. In specific embodiments, the molecule is one which modulates Hu-Asp2 activity. The candidate substance may be a protein or fragment thereof, a small molecule inhibitor, or even a nucleic acid molecule. It may prove to be the case that the most useful pharmacological compounds for identification through application of the screening assay will be compounds that are identified through screening large compound libraries or that are structurally related to other known modulators of APP processing. The active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive. However, prior to testing of such compounds in humans or animal models, it will be necessary to test a variety of candidates to determine which have potential.

Accordingly, the active compounds may include fragments or parts of naturally-occurring compounds or may be found as active combinations of known compounds which are otherwise inactive. Accordingly, the present invention provides screening assays to identify agents which stimulate or inhibit cellular APP processing. It is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents.

It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be polypeptide, polynucleotide, small molecule inhibitors or any other inorganic or organic chemical compounds that may be designed through rational drug design starting from known stimulators or inhibitors of Hu-Asp2 activity and/or APP processing.

The candidate screening assays are simple to set up and perform.

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Thus, in assaying for a candidate substance, after obtaining a cell expressing functional Hu-Asp, one will admix a candidate substance with the cell, under conditions which would allow measurable Hu-Asp2 activity to occur. In this fashion, one can measure the ability of the candidate substance to stimulate the activity of the cell in the absence of the candidate substance. Likewise, in assays for inhibitors after obtaining a cell expressing functional Hu-Asp, the candidate substance is admixed with the cell. In this fashion the ability of the candidate inhibitory substance to reduce, abolish, or otherwise diminish a biological effect mediated by Hu-Asp2 from said cell may be detected.

"Effective amounts" of the substance in certain circumstances are those amounts effective to reproducibly alter a given Hu-Asp2 activity or APP processing in the form of cleavage of the β -secretase cleavage site of the peptide substrates of the present invention in comparison to their normal levels of cleavage in the absence of the candidate substance. Compounds that achieve significant appropriate changes in activity will be used.

Significant changes in Hu-Asp2 activity or function, *e.g.*, as measured using cleavage of the novel Hu-Asp2 peptide substrates of the present invention (see *e.g.*, Example 1) are represented by an increase/decrease in activity of at least about 30%-40%, and most preferably, by changes of at least about 50%, with higher values of course being possible.

The assays described above employing the novel Hu-Asp2 substrates of the invention are amenable to numerous high throughput screening (HTS) methods (For a review see Jayawickreme and Kost, *Curr. Opin. Biotechnol.* 8: 629-634 (1997)). Automated and miniaturized HTS assays are also contemplated as described for example in Houston and Banks *Curr. Opin. Biotechnol.* 8: 734-740 (1997)

There are a number of different libraries used for the identification of small molecule modulators including chemical libraries, natural product libraries and combinatorial libraries comprised of random or designed peptides, oligonucleotides or organic molecules. Chemical libraries consist of structural analogs of known compounds or compounds that are identified as hits or leads via natural product screening or from screening against a potential therapeutic target.

Natural product libraries are collections of products from microorganisms, animals, plants, insects or marine organisms which are used to create mixtures of screening by, *e.g.*, fermentation and extractions of broths from soil, plant or marine organisms. Natural product libraries include polypeptides, non-ribosomal peptides and non-naturally occurring variants thereof. For a review see *Science* 282:63-68 (1998). Combinatorial libraries are composed of large numbers of peptides oligonucleotides or organic compounds as a mixture. They are relatively simple to prepare by traditional automated synthesis methods, PCR cloning or other synthetic methods. Of particular interest will be libraries that include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial and polypeptide libraries. A review of combinatorial libraries and libraries created therefrom, see Myers *Curr. Opin. Biotechnol.* 8: 701-707 (1997). A candidate modulator identified by the use of various libraries described may then be optimized to modulate activity of Hu-Asp2 through, for example, rational drug design.

It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

C. *In Vivo* Assays.

The present invention also encompasses the use of various animal models. Given the disclosure of the present invention, it will be possible to produce non-human models of APP processing in which the normal APP β -secretase cleavage site has been replaced by the peptide substrates of the present invention. This will afford an excellent opportunity to examine the function of Hu-Asp2 in a whole animal system where it is normally expressed. By developing or identifying mice that express the novel β -secretase substrates of the present invention, one can provide models that will be highly predictive of Alzheimer's disease in humans and other mammals, and helpful in identifying potential therapies. Methods of creating such animals are detailed elsewhere in the specification.

Treatment of animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal.

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Administration will be by any route that can be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated are systemic intravenous injection, regional administration via blood, cerebrospinal fluid (CSF) or lymph supply and intratumoral injection.

Determining the effectiveness of a compound *in vivo* may involve a variety of different criteria. Such criteria include, but are not limited to, survival, increased activity level, and improved food intake. Other methods of evaluation include pathological examination, especially of brain tissue, to look for indicia of altered beta secretase activity, such as reduced production of amyloid beta or amyloid beta plaques and reduced atrophy of the brain.

D. Manufacture of Medicaments

The assays of the invention will identify beta secretase modulators that represent candidate therapeutics for treatment of diseases characterized by aberrant levels of beta secretase activity, including Alzheimer's disease. Thus, after identifying modulator agents, the methods of the invention optionally include the additional step or steps of manufacturing/synthesizing the agents, and of formulating the agent into a composition using pharmaceutically acceptable diluents, adjuvants, or carriers. Pharmaceutical compositions are described in greater detail below.

VII. Methods of Making Transgenic Animals

As noted above, particular embodiments of the present invention contemplate the production of transgenic animals comprising an APP mutant having as β -secretase cleavage one of the peptides of the present invention. Exemplary transgenic animals of the present invention are constructed using an expression cassette which includes in the 5' \rightarrow 3' direction of transcription, a transcriptional and translational initiation region associated with expression in the host animal (a promoter region as described below), a DNA encoding a mutant APP gene that

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when expressed as a protein lacks the wild-type or Swedish sequence at the β -secretase cleavage site and instead comprises a sequence of one of the novel Hu-Asp2 peptide substrates of the present invention and/or a selectable marker gene, and a transcriptional and translational termination region functional in the host animal.

The transgenic animals will provide models for the study of the function of Hu-Asp2 and for the development of protocols and regimens for the therapeutic intervention of AD. Preferred animals exhibit characteristics associated with the pathophysiology of AD. Transgenic animals expressing the mutant APP transgenes, recombinant cell lines derived from such animals, and transgenic embryos are all within the purview of this aspect of the invention.

A. Animals Used.

In certain instances, it may be useful to set up a colony of mice for the production of transgenic mice and also for the production of colonies that may be employed for testing the effects of various antidepressant agents. The animals used as a source of fertilized egg cells or embryonic stem cells can be any animal. However, it is generally preferred that the host animal is one which lends itself to multi-generational studies. Transgenic animals may be produced from the fertilized eggs from a number of animals including, but not limited to reptiles, amphibians, birds, mammals, and fish. Within a particularly preferred embodiment, transgenic mice are generated which express a mutant form of APP which comprises a β -secretase cleavage site of one of the peptides of the present invention in place of the wild-type or Swedish mutation sequence. While this section generally discusses mouse colonies, it should be understood that similar considerations will apply to any animals that are employed in or generated according to the present invention.

The animals of a colony for the production and analysis of transgenic animals can be divided into five categories: female animals for matings to produce fertilized eggs; fertile stud males; sterile stud males for producing pseudo-pregnant females; female mice to act as pseudo-pregnant recipients and foster mothers; and transgenic animals, including founder animals and transgenic lines derived from such founders.

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The C57BL/6J inbred strain of females are, to date, the most widely used for mating to produce the fertilized eggs. However, injection often can be performed with F2 hybrids generated from matings of F1 hybrid male and female mice (e.g., C57BL/6J, x CBA/J)F1 female x C57BL/6J, x CBA/J)F1 male). F2 hybrid zygotes from F1 hybrids have been successfully employed to produce transgenic mice. These F1 hybrids include but are not limited to C57BL/6J x CBA/J; C57BL/6J x SJL; C3H/HeJ x C57BL/6J; C3H/HeJ x DBA/2J and C57BL/6J x DBA/2J. Those of skill in the art will be aware of other strains of female mice from which fertile embryos could be generated. In certain embodiments, the transgenic mice of the present invention are created using ES cells from a 129sv mouse strain that are grown in 129sv females. These transgenic mice are then back crossed against C57BL/6 strain. In general, the generation of transgenic animals and their subsequent breeding is more efficient if F2 zygotes are used for microinjection.

A colony for generating transgenic mice also contains fertile studs males. Such males are housed in a separate cages to avoid fighting and injury. These males should be placed in separate cages a few weeks prior to being presented to a superovulated female mouse that will be used for the production fertile eggs. This is necessary because the dominant male will suppress the testosterone and thus sperm production of his littermates. Each superovulated female is placed individually with a stud male.

Sterile males are required for mating to generate pseudo-pregnant recipients and usually are produced by vasectomy. Alternatively genetically sterile studs can be used. Pseudo-pregnant female mice are generated by mating females in natural estrus with vasectomized or genetically sterile males. Pseudo-pregnant females are is competent to receive embryos but do not contain any fertilized eggs. Pseudo-pregnant females are important for making transgenic animals since they serve as the surrogate mothers for embryos that have been injected with DNA or embryonic stem cells. The best pseudo-pregnant recipients are females that have already reared a litter of animals.

Mice that develop from the injected eggs are termed "founder mice". As soon as a founder mouse is identified it is mated to initiate the transgenic line.

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The potential founder transgenic mice are usually screened for the presence or absence of the injected gene by performing a Southern or dot blot hybridization to DNA extracted from the tail. The protein and RNA expression are analyzed and the transgene copy number and/or level of expression are determined using methods known to those of skill in the art. The protein, RNA expression, and transgene copy numbers are determined in weanling animals (4-5 weeks). When a promoter is used which is constitutively active in animals of weanling age and older, it is not expected that there will be changes in levels of transgenic RNA expression animals beyond weanling age. When a developmentally and/or tissue specific promoter is used, the protein levels are monitored to determine expression levels with age. The transgenic animals also are observed for clinical changes. Examples of neurobehavioral disorders for evaluation are poor mating response, agitation, diminished exploratory behavior in a novel setting, and inactivity may well be important behavioral traits associated with depression. Certain transgenic animal models for AD have been described in *e.g.*, U.S. Patent No. 5,877,399; U.S. Patent No. 5,387,742; U.S. Patent No 5,811,633

B. Methods of Making Transgenic Animals.

A transgenic animal can be prepared in a number of ways. A transgenic organism is one that has an extra or exogenous fragment of DNA incorporated into its genome, sometimes replacing an endogenous piece of DNA. At least for the purposes of this invention, any animal whose genome has been modified to introduce a mutation in the native APP, as well as its mutant progeny, are considered transgenic animals. In order to achieve stable inheritance of the extra or exogenous DNA, the integration event must occur in a cell type that can give rise to functional germ cells. The two animal cell types that are used for generating transgenic animals are fertilized egg cells and embryonic stem cells. Embryonic stem (ES) cells can be returned from *in vitro* culture to a "host" embryo where they become incorporated into the developing animal and can give rise to transgenic cells in all tissues, including germ cells. The ES cells are transfected in culture and then the mutation is transmitted into the germline by injecting the cells into an embryo. The animals carrying mutated germ cells are then bred to produce

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transgenic offspring. The use of ES cells to make genetic changed in the mouse germline is well recognized. For a reviews of this technology, those of skill in the art are referred to Bronson and Smithies, *J. Biol. Chem.*, 269(44), 27155-27158, (1994); Torres, *Curr. Top. Dev. Biol.*, 36, 99-114; 1998 and the references contained therein.

Generally, blastocysts are isolated from pregnant mice at a given stage in development, for example, the blastocyst from mice may be isolated at day 4 of development (where day 1 is defined as the day of plug), into an appropriate buffer that will sustain the ES cells in an undifferentiated, pluripotent state. ES cell lines may be isolated by a number of methods well known to those of skill in the art. For example, the blastocysts may be allowed to attach to the culture dish and approximately 7 days later, the outgrowing inner cell mass picked, trypsinized and transferred to another culture dish in the same culture media. ES cell colonies appear 2-3 weeks later with between 5-7 individual colonies arising from each explanted inner cell mass. The ES cell lines can then be expanded for further analysis. Alternatively, ES cell lines can be isolated using the immunosurgery technique (described in Martin, *Proc. Natl. Acad. Sci. USA* 78:7634-7638 (1981)) where the trophectoderm cells are destroyed using anti-mouse antibodies prior to explanting the inner cell mass.

In generating transgenic animals, the ES cell lines that have been manipulated by homologous recombination are reintroduced into the embryonic environment by blastocyst injection (as described in Williams *et al.*, *Cell* 52:121-131 (1988)). Briefly, blastocysts are isolated from a pregnant mouse and expanded. The expanded blastocysts are maintained in oil-drop cultures at 4°C for 10 min prior to culture. The ES cells are prepared by picking individual colonies, which are then incubated in phosphate-buffered saline, 0.5 mM EGTA for 5 min; a single cell suspension is prepared by incubation in a trypsin-EDTA solution containing 1% (v/v) chick serum for a further 5 min at 4°C. Five to twenty ES cells (in Dulbecco's modified Eagle's Medium with 10% (v/v) fetal calf serum and 3,000 units/ml DNAase 1 buffered in 20 mM HEPES [pH 8]) are injected into each blastocyst. The blastocysts are then transferred into pseudo-pregnant recipients and allowed to develop normally. The transgenic mice are identified by coat markers (Hogan *et al.*,

Manipulating the Mouse Embryo, Cold Spring Harbor, N.Y. (1986)). Additional methods of isolating and propagating ES cells may be found in, for example, U.S. Patent No. 5,166,065; U.S. Patent No. 5,449,620; U.S. Patent No. 5,453,357; U.S. Patent No. 5,670,372; U.S. Patent No. 5,753,506; U.S. Patent No. 5,985,659, each incorporated herein by reference.

An alternative method involving zygote injection method for making transgenic animals is described in, for example, U.S. Patent No. 4,736,866, incorporated herein by reference. Additional methods for producing transgenic animals are generally described by Wagner and Hoppe (U.S. Patent No. 4,873,191; which is incorporated herein by reference), Brinster *et al. Proc. Nat'l Acad. Sci. USA*, 82(13) 4438-4442, 1985; which is incorporated herein by reference in its entirety) and in "Manipulating the Mouse Embryo; A Laboratory Manual" 2nd edition (eds., Hogan, Beddington, Costantini and Long, Cold Spring Harbor Laboratory Press, 1994; which is incorporated herein by reference in its entirety).

Briefly, this method involves injecting DNA into a fertilized egg, or zygote, and then allowing the egg to develop in a pseudo-pregnant mother. The zygote can be obtained using male and female animals of the same strain or from male and female animals of different strains. The transgenic animal that is born, the founder, is bred to produce more animals with the same DNA insertion. In this method of making transgenic animals, the new DNA typically randomly integrates into the genome by a non-homologous recombination event. One to many thousands of copies of the DNA may integrate at a site in the genome

Generally, the DNA is injected into one of the pronuclei, usually the larger male pronucleus. The zygotes are then either transferred the same day, or cultured overnight to form 2-cell embryos and then transferred into the oviducts of pseudo-pregnant females. The animals born are screened for the presence of the desired integrated DNA.

DNA clones for microinjection can be prepared by any means known in the art. For example, DNA clones for microinjection can be cleaved with enzymes appropriate for removing the bacterial plasmid sequences, and the DNA fragments electrophoresed on 1% agarose gels in TBE buffer, using standard techniques. The DNA bands are visualized by staining with ethidium bromide, and

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the band containing the expression sequences is excised. The excised band is then placed in dialysis bags containing 0.3 M sodium acetate, pH 7.0. DNA is electroeluted into the dialysis bags, extracted with a 1:1 phenol:chloroform solution and precipitated by two volumes of ethanol. The DNA is redissolved in 1 ml of low salt buffer (0.2 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) and purified on an Elutip-D™ column. The column is first primed with 3 ml of high salt buffer (1 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) followed by washing with 5 ml of low salt buffer. The DNA solutions are passed through the column three times to bind DNA to the column matrix. After one wash with 3 ml of low salt buffer, the DNA is eluted with 0.4 ml high salt buffer and precipitated by two volumes of ethanol. DNA concentrations are measured by absorption at 260 nm in a UV spectrophotometer. For microinjection, DNA concentrations are adjusted to 3 mg/ml in 5 mM Tris, pH 7.4 and 0.1 mM EDTA.

Additional methods for purification of DNA for microinjection are described in Hogan *et al.* Manipulating the Mouse Embryo (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986), in Palmiter *et al.* *Nature* 300:611 (1982); in The Qiagenologist, Application Protocols, 3rd edition, published by Qiagen, Inc., Chatsworth, CA.; and in Sambrook *et al.* Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate. The superovulating females are placed with males and allowed to mate. After approximately 21 hours, the mated females are sacrificed and embryos are recovered from excised oviducts and placed in an appropriate buffer, *e.g.*, Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5 % BSA in a 37.5°C incubator with a humidified atmosphere at 5% CO₂, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Randomly cycling adult female mice are paired with vasectomized males. C57BL/6 or Swiss mice or other comparable strains can be used for this

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purpose. Recipient females are mated at the same time as donor females. At the time of embryo transfer, the recipient females are anesthetized with an intraperitoneal injection of 0.015 ml of 2.5 % avertin per gram of body weight. The oviducts are exposed by a single midline dorsal incision. An incision is then made through the body wall directly over the oviduct. The ovarian bursa is then torn with watchmakers forceps. Embryos to be transferred are placed in DPBS (Dulbecco's phosphate buffered saline) and in the tip of a transfer pipette (about 10 to 12 embryos). The pipette tip is inserted into the infundibulum and the embryos transferred. After the transfer, the incision is closed by two sutures. The pregnant animals then give birth to the founder animals which are used to establish the transgenic line.

VIII. Pharmaceutical Compositions

The modulators of Hu-Asp, APP processing, and/or β -secretase cleavage identified by the present invention may ultimately be formulated into pharmaceutical compositions *i.e.*, in a form appropriate for *in vivo* applications. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render the compositions stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the modulators identified by the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The modulator compositions of the present invention include classic

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pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. The pharmaceutical compositions may be introduced into the subject by any conventional method, *e.g.*, by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, intraocular, retrobulbar, intrapulmonary (*e.g.*, term release); by oral, sublingual, nasal, anal, vaginal, or transdermal delivery, or by surgical implantation at a particular site, *e.g.*, embedded under the splenic capsule, brain, or in the cornea. The treatment may consist of a single dose or a plurality of doses over a period of time.

The modulator compounds identified using the present invention may be prepared for administration as solutions of free base or pharmacologically acceptable salts in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use

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in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

5 Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of
10 preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and
15 agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

20 For oral administration the modulators identified by the present invention may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active
25 ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

30 The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with

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inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups also can be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups also can be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration.

"Unit dose" is defined as a discrete amount of a therapeutic composition dispersed in a suitable carrier. For example, parenteral administration may be carried out with an initial bolus followed by continuous infusion to maintain therapeutic circulating levels of drug product. Those of ordinary skill in the art will readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual patient. More particularly, the dose should be selected to reduce, inhibit, decrease or otherwise abrogate the formation of A β -peptide and more particularly, plaque formation in the brain of a subject exhibiting AD. To this effect, those of skill in the art will be able to employ animal models of AD (*e.g.*, as disclosed in U.S. Patent No. 5,877,399; U.S. Patent No. 5,387,742; U.S. Patent No. 5,811,633) in order to optimize dose

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administration protocols and predict the relevant amounts of pharmaceutical agents required for intervention of AD in a human subject.

The frequency of dosing will depend on the pharmacokinetic parameters of the agents and the routes of administration. The optimal
5 pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. See for example Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publ. Co, Easton PA 18042) pp 1435-1712, incorporated herein by reference. Such formulations may influence the physical state, stability, rate of *in vivo* release and rate of *in vivo*
10 clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface areas or organ size. Further refinement of the calculations necessary to determine the appropriate treatment dose is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and
15 assays disclosed herein as well as the pharmacokinetic data observed in animals or human clinical trials.

Appropriate dosages may be ascertained through the use of established assays for determining blood levels in conjunction with relevant dose-response data. The final dosage regimen will be determined by the attending
20 physician, considering factors which modify the action of drugs, *e.g.*, the drug's specific activity, severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and
25 duration of treatment for specific diseases and conditions.

It will be appreciated that the pharmaceutical compositions and treatment methods of the invention may be useful in fields of human medicine and veterinary medicine. Thus the subject to be treated may be a mammal, preferably human or other animal. For veterinary purposes, subjects include for example,
30 farm animals including cows, sheep, pigs, horses and goats, companion animals such as dogs and cats, exotic and/or zoo animals, laboratory animals including mice rats, rabbits, guinea pigs and hamsters; and poultry such as chickens, turkey, ducks

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and geese.

IX. Examples

The following examples present preferred embodiments and techniques, but are not intended to be limiting. Those of skill in the art will, in light of the present disclosure, appreciate that many changes can be made in the specific materials and methods which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

β -Secretase Assays

Activity assays for Asp2(a) may be performed using synthetic peptide substrates of the present invention as follows. Reactions may be performed in a suitable buffer such as for example, 50 mM 2-[N-morpholino]ethane-sulfonate ("Na-MES," pH 5.5) or 50mM sodium acetate at a pH range of between 4.0 and 6.0, 70 mM peptide substrate, and recombinant Asp2(a) (1-5 μ g protein) for various times at 37°C. An exemplary buffer comprises containing 1% β -octylglucoside. The reaction products are quantified by RP-HPLC using a linear gradient from 0-70 B over 30 minutes (A=0.1% TFA in water, B=0.1% TFA/10% water/90% AcCN). The elution profile is monitored by absorbance at 214 nm.

In such an assay, two product peaks elute before the intact peptide substrate, may be confirmed as the $P_n \dots P_1$ sequence and the $P_1' \dots P_n'$ sequence using for example, Edman sequencing or mass spectrometry. Percent hydrolysis of the peptide substrates is calculated by comparing the integrated peak areas for the two product peptides and the starting material derived from the absorbance at 214 nm. The sequence of cleavage/hydrolysis products may be confirmed using Edman sequencing and MADLI-TOF mass spectrometry.

The specificity of the protease cleavage reaction can be determined by performing the β -secretase assay in the presence of 8 μ M pepstatin A and the presence of a cocktail of protease inhibitors (*e.g.*, 10 μ M leupeptin, 10 μ M E64, and 5 mM EDTA). A β -secretase proteolytic activity insensitive to pepstatin

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(inhibitor of cathepsin D and other aspartyl proteases) or the cocktail (inhibitors of serine proteases, cysteinyl proteases, and metalloproteases, respectively) is indicative of the specificity of the β -secretase activity.

Alternative assays may employ Hu-Asp2(b) expressed in CHO cells and purified using identical conditions for extraction with β -octylglucoside and sequential chromatography over Mono Q[®] and Mono S[®].

An alternative β -secretase assay utilizes internally quenched fluorescent substrates to monitor enzyme activity using fluorescence spectroscopy in a single sample or multiwell format. Each reaction contains for example, 50 mM Na-MES (pH 5.5), a peptide substrate of the present invention (50 μ M) and purified Hu-Asp-2 enzyme. These components are equilibrated to 37 °C for various times and the reaction initiated by addition of substrate. Excitation is performed at 330 nm and the reaction kinetics are monitored by measuring the fluorescence emission at 390 nm. To detect compounds that modulate Hu-Asp-2 activity, the test compounds are added during the preincubation phase of the reaction and the kinetics of the reaction monitored as described above. Activators are scored as compounds that increase the rate of appearance of fluorescence while inhibitors decrease the rate of appearance of fluorescence.

In yet another alternative, the Hu-Asp2 β -secretase assay is conducted using a cell based assay system in which cells such as, for example, HEK293 cells expresses a fusion polypeptide comprising a first portion comprising an Hu-Asp2 substrate peptide of the present invention, a second portion comprising a transmembrane domain that anchors the peptide to the Golgi or endoplasmic reticulum of the cell, and a third portion comprising a reporter gene, such as for example, SEA. The transmembrane domain will act to ensure efficient delivery of the peptide substrate to the cellular environment where it can bind active Hu-Asp2 and be cleaved. The cleavage is detected as a measure of the SEAP activity released into the medium.

30

EXAMPLE 2

Method for Peptide Quantitation by Amino Acid Analysis

The present example provides an exemplary method for the

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quantitation of peptide substrates by amino acid analysis. This method was used to quantify the various substrates of the present invention.

The peptides were quantitated using microwave hydrolyses using CEM Corporation's MDS 2000 microwave oven. Hewlett Packard 300 µl microvials
5 containing approximately 2 µg protein were placed inside a Teflon® PFA digestion vessel (CEM Corporation) containing 4 ml of 6 N HCl (Pierce Constant Boiling) with 0.5% (volume to volume) phenol (Mallinckrodt). The samples were then alternately evacuated and flushed with N₂ five times. The protein was hydrolyzed using a two-stage process. During the first stage 50% of full power (about 650 W) increased the
10 temperature to 100 °C and held that temperature for 1 minute. Immediately following, 75% power increased the temperature to 150 °C and held that temperature for 25 minutes. After cooling, the samples were dried (Savant SpeedVac). Amino acid analyses were performed on samples derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate to yield stable ureas that fluoresce at 395 nm (Waters
15 AccQ•Tag Chemistry Package). The samples were analyzed by reverse phase HPLC on a Hewlett Packard 1100 system and quantification was performed using Hewlett Packard's ChemStation enhanced integrator.

EXAMPLE 3

20 **Testing an Exemplary Peptide of the Invention based on the Structure of Ubiquitin/Oxidized Insulin B Chain**

The present Example describes the production of an exemplary Hu-Asp2 substrate of the present invention and use in an Hu-Asp2 assay of the invention.

25 The substrate KVEANY-EVEGERKK (SEQ ID NO:5) was synthesized using by solid-phase technology employing a Model 433A from Applied Biosystems Inc. The purity of this substrate was assessed by HPLC analysis. Briefly, crude peptide was dissolved in dilute acetic acid, filtered and loaded on preparative reverse phase column (Vydac C-19, 22 x 250 mm, 10 micron) at 4
30 ml/minute 100% A (A:0.1% TFA in water, B:0.07% TFA in acetonitrile). Gradient used was 0→10% B, 10 minutes then 10→50% B, 200 minutes. The column effluent was monitored by absorbance at 220nm and 280nm. Fractions were

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monitored on an analytical reverse phase system (Vydac C18, 4.6 x 250 mm, 5 micron). Solvents and wavelengths as above. The linear gradient for this RP-HPLC was from 0-70% B in 20 minutes at 1.0 ml/min.

5 The chemical authenticity of the peptide was determined using mass spectrometry analysis. More particularly, the chemical authenticity of each peptide was established by mass spectrometry employing a Micromass Platform II mass spectrometer equipped with a Hewlett Packard Series 1050 HPLC system. The identify of the peptide was confirmed by injecting 5 μ l of sample into the flow of 100 μ l/min of 1:1=methanol:water. The mass spectrometer was operating in
10 electrospray ionization mode with needle voltage 3KV, temperature 120°C and cone voltage 30 V.

The above-described peptide was tested as a substrate for Hu-Asp2 activity in a reaction comprising 200mM sodium acetate, pH4.5, 200 μ M substrate, 200nM Hu-Asp2 enzyme at 37 °C. The reaction mixture was allowed to proceed
15 for between 1 and 3 hours. The reaction products were monitored as described in Example 1 and it was found that KVEANY-EVEGERKK (SEQ ID NO:5) was a good substrate for Hu-Asp.

The KVEANY-EVEGERKK (SEQ ID NO:5) peptide was further modified by the insertion of a cys residue was inserted between R and KK to give
20 the peptide KVEANY-EVEGERCKK (SEQ ID NO:6). This peptide was N-terminally biotinylated, and made fluorescent by the covalent attachment of oregon green at the Cys residue. Briefly, for biotinylation, resin-bound peptide was suspended in (approx. 10 ml) dimethylformamide containing diisopropylethylamine (0.15 ml) and reacted with 50 mg EZ-link NHS-LC-biotin (Pierce) at room
25 temperature. The reaction was allowed to proceed for 24 hours (or until ninhydrin negative). The biotinylated peptide was then cleaved in 10 ml trifluoroacetic acid containing ethyl methyl sulfide:anisole:1,2-ethanedithiol (1:3:1; total 5%) for 2 hours at room temperature. The cleavage solution was filtered through a sintered glass funnel and evaporated to near dryness under reduced pressure. The crude
30 peptide was precipitated from the cleavage solution with cold diethyl ether. The precipitated peptide was collected on a sintered glass funnel, washed with diethyl ether, dissolved in dilute acetic and evaporated to dryness under reduced pressure.

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The residue was dissolved in glacial acetic acid and lyophilized.

The purified biotinylated peptide was dissolved in approximately 5 ml (0.1N) NaH_2PO_4 (pH 8.0) that had been previously degassed. 5 mg of Oregon Green 488 maleimide (Molecular Probes) dissolved in 0.5 ml dimethylformamide was then added and the mixture stirred in the dark for 30 minutes at room temperature. Following the reaction, unreacted reagent was quenched by addition of 15 mg of L-cysteine for an additional 30 minutes. The final mixture was then filtered through a sintered glass funnel, acidified by addition of glacial acetic acid, and purified by preparative reverse phase HPLC as described above except the initial elution conditions (100%A) were held constant until the break-through DMF peak eluted.

The resulting compound, Biotin-KVEANY-EVEGERC(oregon green)KK, was tested as a substrate for Hu-Asp2 activity using the following conditions: 200 mM sodium acetate, pH 4.5, 10 μM substrate, 50nM enzyme at 37 °C. The reaction was allowed to proceed for 2hrs and samples withdrawn at several times. The results showed that 20%, 37%, 57%, and 82% cleavage occurred after 15, 30, 60, and 120 minutes, respectively. Do they have comparative #'s for Nature or APP-SW?

Mass spectrometry analysis showed that cleavage had occurred between Tyr and Glu only. This new biotinylated fluorescent peptide had V_{max} and K_m that were at least twice better than a previous biotinylated fluorescent substrate derived from the Swedish Mutant peptide: SEVNL-DAEFR (SEQ ID NO:19).

EXAMPLE 4

APP Constructs Containing Mutated β -Secretase Cleavage Site

This example describes a method for creating mutations of APP using recombinant methods, to introduce sequences of synthetic Asp2 substrates of the invention.

A mammalian cell expression bicistronic construct named 125, which contains a Swedish mutant APP695 and extra dilysine residues at the C-terminus, was used as a starting material to generate novel APP mutants containing sequences

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of the invention. The sequences encoding VNLDA at residues 593 to 597 of this APP695 were replaced with VSYEA (SEQ ID NO:189), ISY-EV (SEQ ID NO:179) or VSYEV (SEQ ID NO:177) using site directed mutagenesis.

5 The site-directed mutagenesis was performed with a commercial kit (QUICKCHANGE, site-directed mutagenesis, Strategene, La Jolla, CA) using the following primers:

10 #ISYEV5 5'-GAG ATC TCT GAA ATT AGT TAT GAA GTA GAA TTC
CGA CAT GAC TCA GG-3' (SEQ ID NO:171)

#ISYEV3 5'-TGA GTC ATG TCG GAA TTC TAC TTC ATA ACT AAT
TTC AGA GAT CTC CTC-3' (SEQ ID NO:172)

15 #VSYEV5 5'-GAG ATC TCT GAA AGT AGT TAT GAA GTA GAA TTC
CGA CAT GAC TCA GG-3' (SEQ ID NO:173)

#VSYEV3 5'-TGA GTC ATG TCG GAA TTC TAC TTC ATA ACT ACT
TTC AGA GAT CTC CTC-3' (SEQ ID NO:174)

20 #VSYEA5 5'-GAG ATC TCT GAA ATT AGT TAT GAA GCA GAA TTC
CGA CAT GAC TCA GG-3' (SEQ ID NO:175)

25 #VSYEA3 5'-TGA GTC ATG TCG GAA TTC TGC TTC ATA ACT AAT
TTC AGA GAT CTC CTC-3' (SEQ ID NO:176)

30 The PCR conditions for performing the site-directed mutagenesis were as follows: denaturing at 95°C for 1 min., annealing at 56°C for 30 sec and amplifying at 68°C for 16 min. The reaction was performed in 50µl of reaction solution which contained 150ng each of primer, 50ng of template DNA, 2.5u of pfu Turbo DNA polymerase and 200µM of dNTP. The amplified products were digested with restriction enzyme Dpn I for 60 minutes followed by transformation. Mini-preps of plasmid DNA were prepared and DNA sequencing was performed to

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identify mutated clones containing the desired modified sequences.

EXAMPLE 5**Cell-Based Testing of Peptide Sequences of the Invention
Designed into Wild-type APP Sequence**

5 The inventors modified APP expression constructs with the mutation
KM-DA₅₉₄₋₅₉₇ (SEQ ID NO:139) to SYEV₅₉₄₋₅₉₇ (SEQ ID NO:140) or SYEA₅₉₄₋₅₉₇
(SEQ ID NO:187) as described in the preceding example 4 to test whether these
mutations would allow an efficient proteolytic processing of the resulting modified
10 (mutant) APP by β -secretase in cells. The resultant expression constructs contained
a CMV promoter to drive expression of a wildtype APP695 sequence modified to
include a C-terminal dilysine, and further modified with the specific mutations
described above. Since APP variants were subcloned into a pIRES-EGFP vector
Clontech, Palo Alto, CA), the efficiency of expression can be monitored by the
presence of green fluorescent signal. After HEK 293 derivative cells that express
15 higher levels of Hu-Asp2 were transfected with different APP variants for 48 hours,
the cell extracts were prepared and analyzed by Western blots transferred from a 4-
12% SDS-PAGE gel. The Hu-Asp2 activity can be monitored by observing the
increase of the β -secretase cleavage product CTF-99 that is recognized by a specific
antibody C8. As shown in FIG. 3, both mutant APP variants were proficiently
20 cleaved by Asp2. Similar to the *in vitro* enzymatic assay, APP with SYEV₅₉₄₋₅₉₇
(SEQ ID NO:140) was cleaved more efficiently than APP with SYEA₅₉₄₋₅₉₇ (SEQ ID
NO:187).

 As one indicator of APP processing, levels of secreted A β from the
medium of the transfected HEK-293 cells were measured. Measurements were by
25 ELISA using antibody 6E10 (Syntek, St. Louis, MO) to capture A β and antibody 208
for A β 40 and antibody 165 for A β 42. Both 208 and 165 were purchased from
Research Foundation of Mental Hygiene (Staten Island, NY), and it was
independently determined that the mutation of D₅₉₆A₅₉₇ to either E₅₉₆A₅₉₇ or E₅₉₆V₅₉₇
(which affects the N-terminus of A β) did not affect ELISA detection of A β peptides.
30 Increased production of secreted A β ₄₀ and A β ₄₂ was observed with cells transfected
with these two mutated forms of APP. Using additional constructs in peptide activity
assays, it was determined that if an Asn residue was substituted in place of a Ser to

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give SEVNY-↓-EVEFR (SEQ ID NO:170), this peptide has an activity that is only 1/5 than that of SEVSY-↓-EVEFR (SEQ ID NO:141; Table 4). This observation reaffirms that amino acid optimization at a certain position depends on the rest of the substrate sequence.

5 Additional cells useful for such assays include

In summary, SEVSY-↓-EVEFR (SEQ ID NO:141) is an excellent APP-modified substrate for Asp2 that gives easily measurable levels of A β in cell-based assays.

10

Example 6

Cell Lines Expressing Mutant APP Forms

The preceding describes assays that employed a transformed HEK-293 cell line. Other useful cell lines include, but not limited to, human HeLa, IMR-32, SK-N-MC, SHY-5Y cells, Chinese Hamster Ovary cells, mouse Neuro-2a cell, and rat PC12 cells. All of these cells are readily available from ATCC. In preferred embodiments, the vector used to introduce the modified APP form also includes an antibiotic resistance gene, *e.g.*, so that the stable cell lines can be selected under G418 resistance conditions. In another preferred embodiment, cell lines are generated that express both mutant APP as described herein and that also recombinantly express Asp2. In one variation, the Asp2 is introduced using a second construct that permits selection under a separate selective antibiotic (*e.g.*, hygromycin selection). Such cell lines are considered especially well suited for high throughput screening for modulators of Asp2-mediated APP processing.

15

20

Example 7**High Throughput Cell-Based Assay for Asp2 Modulators**

Assays such as those described in Example 5 are most useful when they can be conducted quickly, with automation, and with the screening of several samples in parallel. The general procedure described hereafter is applicable for a variety of cell lines including those identified above. Preferably, the cell-based assay is conducted in 96-well format and each candidate compound is measured in duplicates.

About 25,000 cells will be plated onto each well the day before the treatment and grown in normal cell culture conditions (37°C, 5% CO₂).

An appropriate concentration of compound stock is prepared in DMSO. The idea stock solution is about 1,000 times the highest concentration to be tested. On the microplate, compounds are serially diluted 1:3 with DMSO (25µl + 200µl DMSO), and then diluted in media at a ratio 1:5 v/v compound:media (50µl + 200µl media) to yield an intermediate plate used for further dilutions. The intermediate plate is diluted 1:20 (13 µl + 247µl media) to yield concentrations that are ten times the final desired concentrations in duplicate.

To begin the assays, 20 µl of final compound dilution is added to cell plates that contain 180 µl of media, and the plate is put back to the tissue culture incubator for continuing culture. After three hours of incubation, media is collected and the cells are again treated with 180 µl of media plus 20 µl of compound, and then cultured overnight. Media from the cells is collected the next day and assayed to measure evidence of APP processing. For example, Aβ levels are measured from the collected media by ELISA. N-terminal fragments resulting from APP processing also can be measured.

Example 8**Chimeric Protein Comprising MBP and APP****and Asp2 Recognition Sequences of the Invention**

A fusion protein comprising a maltose binding protein (SEQ ID NO:194) with 125 amino acids from APP C-terminus was produced by synthesizing an expression of MBPC125 construct and transforming the construct into an *E. coli*

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host. The MBP (NEB, Beverley, MA) provides a useful detection substrate for detecting cleavage following Asp2 cleavage of substrates of the invention.

Fusions of APP and maltose-binding protein (MBP) are produced essentially as described in U.S. Patent No. 5744346 and U.S. Patent No.

5 5,942,400, (except that β -secretase substrates of the invention are included in the fusion protein). These documents also describe assays, which are run by exposing the fusion polypeptide to β -secretase which cleaves the 125 amino acid portion of APP at the amino-terminus of the β A. The MBP portion may then be captured, and the carboxy-terminus of the APP fragment which is exposed by cleavage with β -
10 secretase may be identified with 192 antibody specific for said terminus. In the present invention, the APP employed in these assays will comprise the peptides of the present invention at the β -secretase cleavage site.

The approach for mutating the β -secretase cleavage site of normal APP (VKM-DA, SEQ ID NO:180) to VSY-EV (SEQ ID NO:177), VSY-DA (SEQ
15 ID NO:178) or ISY-EV (SEQ ID NO:179) is described in a preceding example.

In a related procedure, the MBPC125 construct containing MBPC125-VSYEV was further modified by introducing a stop codon after residues VSY, which corresponds with position P₃P₂P₁. This construct permits one to
20 produce a truncated version of MBPC125 fusion protein that ends at --- VSY. This truncated protein is useful to set up a standard assay curve for measuring Asp2 cleavage products of the MPBC125 proteins because it corresponds to the expected cleavage product. The following pair of primers can be used for the mutagenesis.

#VSYEND5 5'-GAC ATC TCT GAA GTG AGT TAT TAG GCA GAA TTC
25 CGA CAT GAC TCA GG-3 (SEQ ID NO:181)

#VSYEND3 5'-TGA GTC ATG TCG GAA TTC TGC CTA ATA ACT CAC
TTC AGA GAT CTC CTC-3' (SEQ ID NO:182)

PCR procedures as described in a preceding example can be used to introduce the
30 mutation.

EXAMPLE 9

**Creation of Exemplary Fusion Protein Comprising
SEAP, Insulin B chain, and Asp2 Recognition Sequences of the Invention**

The present Example describes a fusion protein in which secreted alkaline phosphatase (SEAP) is fused to either partial or full-length insulin B chain with modified optimal Asp2 cleavage site and the transmembrane domain of
5 Hu-Asp2 (residue 454-477) together with a short C-terminal Flag-tagged tail. The sequence of such peptide/construct depicted in FIG. 1.

When the fusion protein is recombinantly co-expressed with Hu-Asp2 in a cell line or expressed in a cell line with endogenous Hu-Asp2 activity, Hu-Asp2
10 will cleave the fusion protein to release SEAP into the cell medium. Thus, the protease activity may be monitored based on the SEAP activity in the medium.

Generation of the fusion proteins:

To make a fusion protein with secreted alkaline phosphatase as a reporter, the pCMV/SEAP vector DNA from Topix (Bedford, MA) was used. The
15 stop codon TTA of the SEAP coding region was then mutated to an EcoRI site GAATTC by site directed mutagenesis. The mutated plasmid was then digested with EcoRI completely and treated with calf intestine alkaline phosphatase to dephosphorylate the vector DNA. This treated vector DNA was used for the
20 subsequent insertion of DNA fragments covering the components 2 to 4 in Table 4 below.

A single chain cDNA fragment encoding human insulin β -chain was synthesized and the double strand DNA was produced by PCR amplification using this chain as a template. The 5'-primer contains an overhang of EcoRI cleavage
25 site. The 3'-primer contains a Bgl II site. The transmembrane domain of human Asp2 was also produced by PCR amplification with 3'-primer overhanged with a flag-tag coding sequences and an EcoRI cloning site and 5'-primer with a Bgl II site. The two DNA fragments were digested with restriction enzymes EcoRI and BglII and subsequently ligated into the above vector DNA. Various peptides of the
30 present invention spanning the β -secretase cleavage site were generated by site-directed mutagenesis.

Table 7

Component 1 (reporter)	Component 2 (cleavage site)	Component 3 (membrane target sequence)	Component 4 (cytosol tail)
SEAP Sequence (residue 1-506)	VEANY-EVEGE (SEQ ID NO:184)	Asp2 transmembrane	flag tag sequence
luciferase (full length)	VEANY-AVEGE (SEQ ID NO:185)	APP residue 598-661	DYKDDDDK (SEQ ID NO:186)
CAT (full length)			APP residue 662-695
β -galactosidase (full length)		galactosyltransferase (residue 4-27)	
		Asp1 transmembrane domain (residue 470-492)	
		sialyltransferase (residue 10-33)	
		syntaxin 6 (residue 261-298)	
		acetyl glucosaminyl transferase (residue 7-29)	

Another type of fusion protein contemplated employs the C-terminal region of APP. A PCR product corresponding to the C-terminal 97 amino acids of APP (residue 598-695) is generated. There is a natural EcoRI site at the residue 598 and an EcoRI site may be included at the 3'-primer. The PCR product is digested with EcoRI and then inserted into the SEAP vector. DNA sequences coding the various Hu-Asp2 peptide substrates described in the present invention are inserted into the β -secretase cleavage region of the APP by the site directed mutagenesis approach.

Example 10

Generation of constructs for producing mutant APP transgenic mice.

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As described above, transgenic animals comprising the synthetic beta secretase recognition sequences of the present invention comprise useful animal models for disease states. For example, these animals will be useful models for Alzheimer's Disease. This example describes a suitable construct for introducing
5 such sequences into mice.

Materials and methods for creating transgenic mice are now well known and have been described in the literature, and are easily adapted to make transgenic mice of the invention. For example, U.S. Patent No. 5,877,399 (incorporated herein by reference) describes transgenic mice that express an APP
10 Swedish mutation. Additional transgenic mice are described in U.S. Patent 5,387,742. It is contemplated that techniques similar to those described in the aforementioned patents may be used in conjunction with the teachings of the present invention to yield mouse models. Specifically, the constructs for producing the transgenic mice in the present invention will comprise an APP that has been mutated
15 (preferably at or near the codons for its natural β -secretase recognition sequence) to include sequence encoding one of the peptides of the present invention.

In specific exemplary transgenic mice the mutant APP will have a replacement of the four amino acids surrounding the β -secretase cleavage site in APP, *i.e.*KM-↓-DA....(SEQ ID NO:139), with ...SY-↓-EV...(SEQ ID NO:140).
20 In other specific embodiments, it is contemplated that the mutant APP (*e.g.*, mutant of human APP695) will comprise any of the peptides of the present invention with a C-terminal dilysine addition. A preferred construct is a mutant human APP695 with a C-terminal dilysine addition and with the SYEV beta secretase recognition sequence of the invention.

25 A commercially available murine prion (PrP) protein vector from Life Tech was selected for experimentation. The vector was digested with Xho I and blunt-ended. Attr sequence was blunt cloned into the Xho I site of PrP vector and orientation selected by sequence analysis.

The beta secretase substrate selected for introduction into the mouse
30 comprised human APP695 with a C-terminal dilysine addition and with the SYEV beta secretase recognition sequence of the invention (APP-kk-syev, SEQ ID

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NO:183). This modified APP sequence was cloned into pDONR201 and the recombination reaction inserted the APP-kk-syev sequence into the PrP vector downstream of the mouse prion promoter and upstream of mouse PRP 3' flanking sequence.

5 Using homologous recombination techniques known in the art and/or described above, this construct can be used to introduce the modified APP sequence into murine embryonic stem cells, and the cells can be used to generate transgenic mice harboring an APP containing the artificial beta secretase substrate sequences of the present invention.

10 It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples.

 Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the invention. The entire disclosure of all publications cited herein are hereby
15 incorporated by reference.

WHAT IS CLAIMED IS:

1. An isolated peptide comprising a sequence of at least four amino acids defined by formula $P_2P_1-P_1'P_2'$ wherein

5 P_2 is a charged amino acid, a polar amino acid, or an aliphatic amino acid but is not an aromatic amino acid;
 P_1 is an aromatic amino acid or an aliphatic amino acid but not a polar amino acid or a charged amino acid;
 P_1' is a charged amino acid, or aliphatic amino acid, or a polar amino
10 acid but is not an aromatic amino acid;
 P_2' is an uncharged aliphatic polar amino acid or an aromatic amino acid; and

wherein said peptide is cleaved between P_1 and P_1' by a human aspartyl
15 protease encoded by the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:3 and said peptide does not comprise the corresponding $P_2P_1-P_1'P_2'$ portion of amino acid sequences depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID
20 NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40.

2. The isolated peptide of claim 1, comprising an amino acid sequence defined by formula $P_2P_1-P_1'P_2'P_3'$, wherein P_3' is any amino acid, and wherein said
25 peptide does not comprise the corresponding $P_2P_1-P_1'P_2'P_3'$ portion of amino acid sequences depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID
30 NO:40.

3. The isolated peptide of claim 1, comprising an amino acid sequence defined by formula $P_3P_2P_1-P_1'P_2'P_3'$, wherein P_3 is an uncharged polar amino acid, an uncharged aliphatic amino acid, or an aromatic amino acid, and wherein said said peptide does not comprise the corresponding $P_3P_2P_1-P_1'P_2'P_3'$ portion of amino acid sequences depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40.
4. The isolated peptide of claim 3, comprising an amino acid sequence defined by formula $P_4P_3P_2P_1-P_1'P_2'P_3'$, wherein said P_4 is a charged amino acid, a polar amino acid or an aliphatic amino acid but not an aromatic amino acid and said peptide does not comprise the corresponding $P_4P_3P_2P_1-P_1'P_2'P_3'$ portion of amino acid sequences depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40.
5. The isolated peptide of any one of claims 2 through 4, further comprising an amino acid at position P_4' immediately to the carboxy-terminal position of P_3' wherein said P_4' is any amino acid said, and wherein the peptide does not comprise the corresponding $P_3P_2P_1-P_1'P_2'P_3'P_4'$ portion of amino acid sequences depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40.
6. The isolated peptide of claim 1, wherein said P_2 is an amino acid selected from the group consisting of N, L, K, S, G, T, D, A, Q and E.

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7. The isolated peptide of claim 1, wherein said P_1 is an amino acid selected from the group consisting of Y, L, M, Nle, F, and H.
- 5 8. The isolated peptide of claim 1, wherein said P_1' is an amino acid selected from the group consisting of E, A, D, M, Q, S and G.
9. The isolated peptide of claim 1, wherein said P_2' is an amino acid selected from the group consisting of V, A, N, T, L, F, and S.
- 10 10. The isolated peptide of claim 2, wherein said P_3' is an amino acid selected from the group consisting of E, G, F, H, cysteic acid and S.
11. The isolated peptide of claim 3, wherein said P_3 is an amino acid selected from the group consisting of A, V, I, S, H, Y, T and F.
- 15 12. The isolated peptide of claim 4, wherein said P_4 is an amino acid selected from the group consisting of E, G, I, D, T, cysteic acid and S.
13. The isolated peptide of any one of claims 4-12, wherein said P_4' is an amino acid selected from the group consisting of F, W, G, A, H, P, G, N, S, and E.
- 20 14. The isolated peptide of any one of claims 1 through 13 further comprising a first label.
- 25 15. The isolated peptide of claim 14 further comprising a second label.
16. An isolated peptide according to any one of claims 1-13, further comprising a detectable label and a quenching moiety, wherein cleavage of the peptide between P_1 and P_1' separate the quenching moiety from the label to permit detection of the label.
- 30

17. The isolated peptide of claim 10 or 12, wherein said cysteic acid further comprises a covalently attached label.
- 5 18. The isolated peptide of any one of claims 1-17, wherein the rate of cleavage of said peptide by said human aspartyl protease is greater than the rate of cleavage of a polypeptide comprising the human APP β -secretase cleavage sequence: SEVKM-DAEFR (SEQ ID NO:20).
- 10 19. The isolated peptide of any one of claims 1-17, wherein the rate of cleavage of said peptide by said human aspartyl protease is greater than the rate of cleavage of a polypeptide comprising the human APP Swedish KM \rightarrow NL mutation, β -secretase cleavage sequence SEVNL-DAEFR (SEQ ID NO:19).
- 15 20. The isolated peptide of claim 1, wherein said peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:5; SEQ ID NO:6; SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:9; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:12; SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15; SEQ ID NO:16; SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:120; SEQ ID NO:133; SEQ ID NO:134; SEQ ID NO:135; SEQ ID NO:136; SEQ ID NO:137; SEQ ID NO:138; SEQ ID NO:141; SEQ ID NO:143; SEQ ID NO:144; SEQ ID NO:145; SEQ ID NO:147; SEQ ID NO:148; SEQ ID NO:149; SEQ ID NO:150; SEQ ID NO:151; SEQ ID NO:152; SEQ ID NO:153; SEQ ID NO:154; SEQ ID NO:155; SEQ ID NO:156; SEQ ID NO:157; SEQ ID NO:158; SEQ ID NO:159; SEQ ID NO:160; SEQ ID NO:161; SEQ ID NO:162; SEQ ID NO:163; SEQ ID NO:164; SEQ ID NO:165; SEQ ID NO:166; SEQ ID NO:167; SEQ ID NO:168; SEQ ID NO:169; SEQ ID NO:190; SEQ ID NO:191; SEQ ID NO:192 and SEQ ID NO:193.
- 20 25 30 21. An isolated peptide comprising a sequence of at least four amino acids

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defined by formula $P_2P_1--P_1'P_2'$, wherein:

P_2 comprises an amino acid selected from the group consisting of N, S, and D;

5 P_1 comprises an amino acid selected from the group consisting of Y, L, and Nle;

P_1' comprises an amino acid selected from the group consisting of E, A, and D;

P_2' comprises an amino acid selected from the group consisting of A and V;
and

10 wherein a human Aspartyl protease encoded by the nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3 (Hu-Asp2) cleaves said peptide between P_1 and P_1' ;

with the proviso that if $P_1'P_2'$ comprise the sequence DA, P_2P_1 do not comprise the sequences NL or NNle.

15

22. An isolated peptide according to claim 21, wherein the peptide amino acid sequence consists of 4-50 amino acids..

20 23. An isolated peptide according to claim 21, wherein the Hu-Asp2 cleaves the peptide at a rate greater than the Hu-Asp2 cleaves a corresponding peptide having the $P_2P_1--P_1'P_2'$ amino acid sequence KMDA.

25 24. An isolated peptide according to claim 21, wherein the Hu-Asp2 cleaves the peptide at a rate greater than the Hu-Asp2 cleaves a corresponding peptide having the $P_2P_1--P_1'P_2'$ amino acid sequence KMDA.

25. A peptide according to claim 21, further comprising a label.

30 26. A peptide according to claim 21, further comprising a label and a quenching moiety that quenches the label, wherein the label and quenching moiety are

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attached on opposite sides of the P_1 - P_1 peptide bond, whereby cleavage of the P_1 - P_1 peptide bond separates the label and quenching moiety.

- 5 27. A polypeptide comprising a peptide sequence according to claim 21, and further comprising a transmembrane domain to localize the polypeptide to a cellular membrane when the polypeptide is expressed in a eukaryotic cell.
- 10 28. A polypeptide comprising a peptide according to any one of claims 1 through 20 and further comprising a transmembrane domain amino acid sequence.
29. A polypeptide according to claim 28, wherein the peptide is N-terminal to the transmembrane domain.
- 15 30. The polypeptide of claim 28 or 29, wherein the peptide and the transmembrane domain are separated by a linker.
31. The polypeptide of claim 30, wherein said linker is a peptide linker comprising between about 20 to about 40 amino acids.
- 20 32. The polypeptide according to any one of claims 28-31, wherein said transmembrane domain anchors said polypeptide to an intracellular membrane selected from the group consisting of the Golgi or the endoplasmic reticulum.
- 25 33. The fusion protein of any of claims 28-32, wherein said transmembrane domain is selected from the group consisting of the transmembrane domain of galactosyltransferase, the transmembrane domain of sialyl transferase; the transmembrane domain of human aspartyl transferase 1; the
- 30 transmembrane domain of human aspartyl transferase 2; the transmembrane

domain of syntaxin 6; the transmembrane domain of ubiquitin; the transmembrane domain of insulin B chain and the transmembrane domain of APP.

- 5 34. The polypeptide according to any one of claims 28-32, further comprising a reporter protein amino acid sequence.
35. The polypeptide of claim 34, wherein said reporter protein is selected from the group consisting of luciferase; alkaline phosphatase; β -galactosidase; β -glucuronidase; green fluorescent protein; chloramphenical acetyl transferase;
- 10 36. A polynucleotide comprising a nucleotide sequence that encodes a polypeptide according to any one of claims 20-35.
37. A polynucleotide comprising a nucleotide sequence that encodes a peptide according to any one of claims 1-27.
- 15 38. A vector comprising a polynucleotide according to claim 36.
39. A vector comprising a polynucleotide according to claim 37.
- 20 40. A vector according to claim 38 wherein said polynucleotide is operably linked to a promoter to promote expression of the fusion protein encoded by the polynucleotide in a host cell.
- 25 41. A host cell transformed or transfected with a polynucleotide according to claim 36.
42. A host cell transformed or transfected with a vector according to any one of claims 38-40.
- 30

43. A method for assaying for modulators of β -secretase activity, comprising the steps of:
- (a) contacting a first composition with a second composition both in the presence and in the absence of a putative modulator compound,
5 wherein the first composition comprises a mammalian β -secretase polypeptide or biologically active fragment thereof, and wherein the second composition comprises a substrate, wherein said substrate comprises a peptide according to any of claims 1 through 26 or a polypeptide according to any of claims 27-35;
 - 10 (b) measuring cleavage of the substrate peptide in the presence and in the absence of the putative modulator compound; and
 - (c) identifying modulators of β -secretase activity from a difference in cleavage in the presence versus in the absence of the putative
15 modulator compound, wherein a modulator that is a β -secretase antagonist reduces such cleavage and a modulator that is a β -secretase agonist increases such cleavage.
44. The method of claim 43, wherein said first composition comprises a purified human Asp2 polypeptide.
20
45. The method of claim 43, wherein said first composition comprises a soluble fragment of a human Asp2 polypeptide that retains Asp2 β -secretase activity.
46. The method of claim 45, wherein said soluble fragment is a fragment lacking
25 an Asp2 transmembrane domain.
47. A method according to claim 43, wherein the β -secretase polypeptide of the first composition comprises a polypeptide purified and isolated from a cell transformed or transfected with a polynucleotide comprising a nucleotide
30 sequence that encodes the β -secretase polypeptide.

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- 48 A method according to claim 43, wherein the polypeptide of the first composition is expressed in a cell transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes the polypeptide, and wherein the measuring step comprises measuring APP processing activity of the cell.
- 5
49. The method claim of any of claims 43-48, further comprising a step of treating Alzheimer's Disease with an agent identified as an inhibitor of Hu-Asp2.
- 10
50. A β -secretase modulator identified according to the method of any of claims 43-48.
51. A method of inhibiting β -secretase activity *in vivo* comprising a step of administering a modulator according to claim 50 that is a β -secretase antagonist to a mammal in an amount effective to inhibit β -secretase in cells of said mammal.
- 15
52. A method of producing a substrate for a β -secretase assay comprising:
growing a host cell transformed or transfected with a vector of claim
40 in a manner allowing expression of said polypeptide.
- 20
53. The method of claim 52, further comprising purifying said polypeptide.
54. The method of claim 52, wherein said host cell is selected from the group consisting of a mammalian host cell, a bacterial host cell and a yeast host cell.
- 25
55. A pharmaceutical composition comprising a modulator of claim 50 and a pharmaceutically acceptable carrier.
- 30

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56. A method of treating a disease or condition characterized by an abnormal β -secretase activity comprising administering to a subject in need of treatment a pharmaceutical composition of claim 55.
- 5 57. A use of a modulator identified according to the method claim 43 in the manufacture of a medicament for the treatment of Alzheimer's Disease.
58. A method for identifying agents that inhibit the activity of human Asp2 aspartyl protease (Hu-Asp2), comprising the steps of:
- 10 (a) contacting a peptide of any of claims 1 through 26 or a polypeptide of any of claims 27-35 and a composition comprising an Hu-Asp2 activity in the presence and absence of a test agent;
- (b) determining the cleavage of said peptide or polypeptide
15 between said P_1 and P_1' by said Hu-Asp2 in the presence and absence of the test agent; and
- (c) comparing said cleavage activity of the Hu-Asp2 in the
20 presence of the test agent to the activity in the absence of the test agent to identify an agent that inhibits said cleavage by the Hu-Asp2, wherein reduced activity in the presence of the test agent identifies an agent that inhibits Hu-Asp2 activity.
59. A method according to claim 58, wherein the Hu-Asp2 is a recombinant Hu-Asp2 purified and isolated from a cell transformed or transfected with a
25 polynucleotide comprising a nucleotide sequence that encodes Hu-Asp2.
60. A method according to claim 58,
wherein the Hu-Asp2 is expressed in a cell, wherein the contacting
comprises growing the cell in the presence and absence of the test
30 agent, and

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wherein the determining step comprises measuring cleavage of said peptide or fusion protein.

- 5 61. A method according to claim 60, wherein the cell further comprises a polynucleotide encoding the polypeptide, and wherein the contacting step comprises growing the cell under conditions in which the cell expresses the polypeptide.
- 10 62. A method according to claim 60 or 61, wherein the cell is a human embryonic kidney cell line 293 cell.
63. A method according to any one of claims 59-62 wherein the nucleotide sequence is selected from the group consisting of:
- 15 (a) a nucleotide sequence encoding the Hu-Asp2(a) amino acid sequence set forth in SEQ ID NO: 2;
- (b) a nucleotide sequence encoding the Hu-Asp2(b) amino acid sequence set forth in SEQ ID NO: 4;
- 20 (c) a nucleotide sequence encoding a fragment of Hu-Asp2(a) (SEQ ID NO: 2) or Hu-Asp2(b) (SEQ ID NO: 4), wherein said fragment exhibits aspartyl protease activity characteristic of Hu-Asp2(a) or Hu-Asp2(b); and
- (d) a nucleotide sequence of a polynucleotide that hybridizes under stringent hybridization conditions to a Hu-Asp2-encoding polynucleotide selected from the group consisting of
- 25 SEQ ID NO: 1 and SEQ ID NO: 3.
58. A method for identifying agents that modulate the activity of Asp2 aspartyl protease, comprising the steps of:
- 30 (a) contacting an Asp2 aspartyl protease and a peptide of any of claims 1 through 26 or a polypeptide of any of claims 27 through 35 in the

- presence and absence of a test agent, wherein the Asp2 aspartyl protease is encoded by a nucleic acid molecule that hybridizes under stringent hybridization conditions to a Hu-Asp2-encoding polynucleotide selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 3;
- 5 (b) determining the cleavage of said peptide or fusion protein between said P₁ and said P₁' site by said Asp2 in the presence and absence of the test agent; and
- (c) comparing the cleavage activity of said Asp2 in the presence of the test agent to the cleavage activity in the absence of the agent to identify agents that modulate the activity of the polypeptide, wherein a modulator that is an Asp2 inhibitor reduces said cleavage and a modulator that is an Asp2 agonist increases said cleavage.
- 10
- 15 65. A method according to 58 or 64, further comprising a step of treating Alzheimer's Disease with an agent identified as an inhibitor of Hu-Asp2.
67. A method for identifying agents that inhibit the activity of human Asp2 aspartyl protease (Hu-Asp2), comprising the steps of:
- 20 (a) growing a cell in the presence and absence of a test agent, wherein the cell expresses an Hu-Asp2 and expresses a protein comprising a peptide of any of claims 1 through 26 or a polypeptide of any of claims 27 through 35;
- (b) determining the determining the cleavage of said protein at the site between said P₁ and P₁' in said cell in the presence and absence of the test agent; and
- 25 (c) comparing said cleavage activity in the presence of the test agent to the cleavage activity in the absence of the test agent to identify an agent that inhibits the activity of Hu-Asp2, wherein reduced cleavage activity in the presence of the test
- 30

agent identifies an agent that inhibits Hu-Asp2 activity.

- 5 68. A method according to claim 67, wherein the host cell has been transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes a Hu-Asp2, wherein said nucleotide sequence is selected from the group consisting of:
- (a) a nucleotide sequence encoding the Hu-Asp2(a) amino acid sequence set forth in SEQ ID NO: 2;
 - 10 (b) a nucleotide sequence encoding the Hu-Asp2(b) amino acid sequence set forth in SEQ ID NO: 4;
 - (c) a nucleotide sequence encoding a fragment of Hu-Asp2(a) (SEQ ID NO: 2) or Hu-Asp2(b) (SEQ ID NO: 4), wherein said fragment exhibits aspartyl protease activity characteristic of Hu-Asp2(a) or Hu-Asp2(b); and
 - 15 (d) a nucleotide sequence of a polynucleotide that hybridizes under stringent hybridization conditions to a Hu-Asp2-encoding polynucleotide selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 3.
- 20 68. A method according to any one of claims 66-67, further comprising a step of treating Alzheimer's Disease with an agent identified as an inhibitor of Hu-Asp2 according to steps (a)-(c).
- 25 69. The use of an agent identified as an inhibitor of Hu-Asp2 according to any one of claims 66-67 in the manufacture of a medicament for the treatment of Alzheimer's Disease.
- 30 70. A kit for performing a β -secretase assay comprising a β -secretase substrate comprising a peptide according to any of claims 1 through 27 and a β -secretase enzyme.

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71. The kit of claim 70, wherein said β -secretase substrate is a polypeptide according to any of claims 28-35.
- 5 72. The kit of claim 70 or 71, further comprising reagents for detecting the cleavage of said peptide or fusion protein.
73. An isolated peptide comprising a sequence of at least 10 amino acids having the sequence SEISY-EVEFR (SEQ ID NO:152).
- 10 74. The isolated peptide of claim 73, wherein said peptide comprises at least 3 amino acids immediately to the carboxy-terminal of SEISY-EVEFR (SEQ ID NO:152).
- 15 75. The isolated peptide of claim 73, wherein said peptide comprises at least 3 amino acids immediately to the amino-terminal of SEISY-EVEFR (SEQ ID NO:152).
- 20 76. The isolated peptide of claim 73, wherein said peptide comprises at least 5 amino acids immediately to the carboxy-terminal of SEISY-EVEFR (SEQ ID NO:152).
- 25 77. The isolated peptide of claim 73, wherein said peptide comprises at least 5 amino acids immediately to the amino-terminal of SEISY-EVEFR (SEQ ID NO:152).
78. The isolated peptide of claim 73, wherein said peptide comprises at least 10 amino acids immediately to the amino-terminal of SEISY-EVEFR (SEQ ID NO:152).
- 30 79. The isolated peptide of claim 73, wherein said peptide comprises at least 13

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amino acids.

80. The isolated peptide of claim 73, wherein said peptide comprises at least 15 amino acids.

5

81. The isolated peptide of claim 73, wherein said peptide comprises at least 20 amino acids.

82. The isolated peptide of claim 73, wherein said peptide comprises at least 50 amino acids.

10

FIG. 1A

ATGCTGCTGCTGCTGCTGGGCTGAGGCTACAGCTCTCCCTGGGCATCATCCCA
M L L L L L L L L G L R L Q L S L G I I P
GTTGAGGAGGAGAACCCGCACTTCTGGAACCGGAGGACCGAGGCCCTGGGTGCCGCC
V E E N P D F W N R E A A E A L G A A
AAGAAGCTGCAGCCTGCACAGACAGCCGCCAAGAACCTCATCTTCTTGGGCGATGGG
K K L Q P A Q T A A K N L I I F L G D G

ATGGGGTGTCTACGGTGACAGCTGCCAGGATCCTAAAGGGCAGAAAGGACAAACTG
M G V S T V T A A R I L K G Q K K D K L

GGGCTGAGATACCCCTGGCCATGGACCGCTTCCCATATGTGGCTCTGTCCAAGACATAC
G P E I P L A M D R F P Y V A L S K T Y

AATGTAGACAAACATGTGCCAGACAGTGGAGCCACAGCCACGGCCTACCTGTGCGGGTC
N V D K H V P D S G A T A T A Y L C G V

AAGGGCAACTCCAGACCATTTGGCTTGAGTGCAGCCCGCTTAAACCAGTGCAACACG
K G N F Q T I G L S A A A R F N Q C N T

ACACGGGCAACGAGGTCACTCTCGTGATGAATCGGGCCAAGAAAGCAGGGAAGTCAGTG
T R G N E V I S V M N R A K A G K S V

Fig. 1A

Fig. 1B

FIG. 1B

GGAGTGGTAACCAACACACGAGTGCAGCAGCCCTCGCCAGCCGGCACCTACGCCACACG
G V V T T T R V Q H A S P A G T Y A H T

GTGAACCGCAACTGGTACTCGGACGCCGACGTGCTGCTCGGCCCGCCAGGAGGGTGC
V N R N W Y S D A D V P A S A R Q E G C

CAGGACATCGCTACGCAGCTCATCTCCAACATGGACATTGACGTGATCCTAGTGGAGGC
Q D I A T Q L I S N M D I D V I L G G G

CGAAAGTACATGTTTCCCATGGGAACCCAGACCCCTGAGTACCCAGATGACTACAGCCAA
R K Y M F P M G T P D P E Y P D D Y S Q

GGTGGGACCAGGCTGGACGGGAAGAATCTGGTGCAGGAATGGCTGGCGAAGCGCCAGGGT
G G T R L D G K N L V Q E W L A K R Q G

GCCCGGTATGTGTGGAACCGCACTGAGCTCATGCAGGCTTCCCTGGACCCCGTCTGTGACC
A R Y V W N R T E L M Q A S L D P S V T

CATCTCATGGTCTCTTTGAGCCCTGGAGACATGAAATACGAGATCCACCGAGACTCCACA
H L M G L F E P G D M K Y E I H R D S T

Fig. 1C

FIG. 1C

CTGGACCCCTCCCTGATGGAGATGACAGAGGCTGCCCTGCGCCTGTGAGCAGGAACCCC
L D P S L M E M T E A A L R L L S R N P

CGCGGCTTCTTCTTCGTGGAGGGTGGTCGCAATCGACCATGGTCATCATGAAGCAGG
R G F F L F V E G G R I D H G H E S R

GCTTACCGGCACTGACTGAGACGATCATGTTCGACGACGCCATTGAGAGGGCGGCCAG
A Y R A L T E T I M F D D A I E R A G Q

CTCACGAGCGAGGACACGCTGAGCCTCGTCACTGCCGACCACTCCCAACGCTCTCTCC
L T S E E D T L S L V T A D H S H V F S

TTCGGAGGCTACCCCTGCGAGGGAGCTCCATCTTCGGGCTGGCCCTGGCAAGGCCCGG
F G G Y P L R G S S I F G L A P G K A R

GACAGAAGGCCTACCGTCCCTCCTATACGGAACGGTCCAGGCTATGTGCTCAAGGAC
D R K A Y T V L L Y G N G P G Y V L K D

GGCGCCCGCGGATGTTACCGAGAGCGGAGCGGAGCCCCGAGTATCGGCAGCAGTCA
G A R P D V T E S E S G S P E Y R Q Q S

Fig. 1D

FIG. 1D

GCAGTGCCCTGGACGAAGAGACCCACGCGGCGAGACGTGGCGGTGTTCCGCGCGGC
 A V P L D E E T H A G E D V A V F A R G

CCGAGGCGCACCTGGTTCACGGCGGTGCAGGAGCAGACCTTCATAGCGCACGTCATGGCC
 P Q A H L V H G V Q E Q T F I A H V M A

TTCGCGCCTGGAGCCCTACACCGCCTGCGACCTGGCGCCCCCGCGCACCAAC
 F A A C L E P Y T A C D L A P P A G T T

GACGCGCGCACCCAGGTAACCTATGAAGTTGAATTCCGAAGAGCACTCTACGTAGAGGT
 D A A H P G N Y E V E P R R A L Y V E G

GAAAGAGGATTCTTCTACACTCCAAAGGCACCTCTACCTCGTAGAGGGTGAAAGAGGATTC
 E R G F F Y T P K A L Y L V E G E R G F

TTCTACACTAGTCTCATGACCATAGCCTATGTTCATGGCTGCCATCTGGCGCCCTCTTCATG
 F Y T S L M T I A Y V M A A I C A L F M

CTGCCACTCTGCCTCATGGTGGACTACAAGGATGATGACAAGTAG
L P L C L M V D Y K D D D K *

Fig. 2

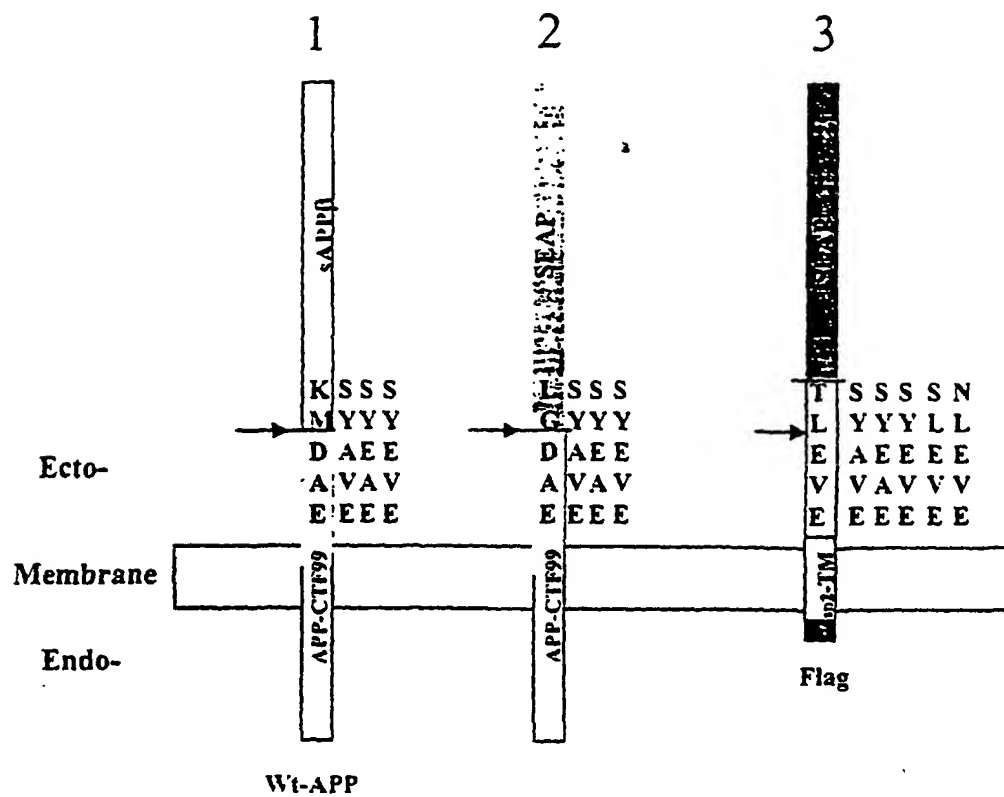


FIG. 2

Fig. 3

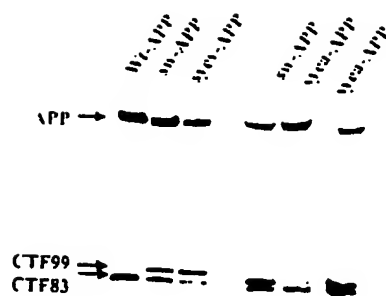


FIG. 3

- 1 -

SEQUENCE LISTING

<110> Yan, Riqiang
Tomasselli, Alfredo G.
Gurney, Mark E.
Emmons, Thomas L.
Bienkowski, Mike J.
Heinrikson, Robert L.

<120> SUBSTRATES AND ASSAYS FOR BETA-SECRETASE ACTIVITY

<130> 29915/00281

<140> 60/219,795

<141> 2000-07-19

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<211> 501

<212> PRT

<213> Homo sapiens

<400> 2

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Gly Leu Gly Gly Ala Pro Leu Gly Leu Arg Leu Pro Arg Glu Thr Asp
          35           40           45

Glu Glu Pro Glu Glu Pro Gly Arg Arg Gly Ser Phe Val Glu Met Val
 50           55           60

Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr Val Glu Met Thr
 65           70           75           80

Val Gly Ser Pro Pro Gln Thr Leu Asn Ile Leu Val Asp Thr Gly Ser
          85           90           95

Ser Asn Phe Ala Val Gly Ala Ala Pro His Pro Phe Leu His Arg Tyr
          100          105          110

Tyr Gln Arg Gln Leu Ser Ser Thr Tyr Arg Asp Leu Arg Lys Gly Val
          115          120          125

Tyr Val Pro Tyr Thr Gln Gly Lys Trp Glu Gly Glu Leu Gly Thr Asp
 130          135          140

Leu Val Ser Ile Pro His Gly Pro Asn Val Thr Val Arg Ala Asn Ile
 145          150          155          160

Ala Ala Ile Thr Glu Ser Asp Lys Phe Phe Ile Asn Gly Ser Asn Trp
          165          170          175

Glu Gly Ile Leu Gly Leu Ala Tyr Ala Glu Ile Ala Arg Pro Asp Asp
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Ser Leu Glu Pro Phe Phe Asp Ser Leu Val Lys Gln Thr His Val Pro
          195          200          205

Asn Leu Phe Ser Leu His Leu Cys Gly Ala Gly Phe Pro Leu Asn Gln
          210          215          220

Ser Glu Val Leu Ala Ser Val Gly Gly Ser Met Ile Ile Gly Gly Ile
 225          230          235          240

Asp His Ser Leu Tyr Thr Gly Ser Leu Trp Tyr Thr Pro Ile Arg Arg
          245          250          255

Glu Trp Tyr Tyr Glu Val Ile Ile Val Arg Val Glu Ile Asn Gly Gln
          260          265          270

Asp Leu Lys Met Asp Cys Lys Glu Tyr Asn Tyr Asp Lys Ser Ile Val
          275          280          285

Asp Ser Gly Thr Thr Asn Leu Arg Leu Pro Lys Lys Val Phe Glu Ala
 290          295          300

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 Thr Asn Gln Ser Phe Arg Ile Thr Ile Leu Pro Gln Gln Tyr Leu Arg
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 Pro Val Glu Asp Val Ala Thr Ser Gln Asp Asp Cys Tyr Lys Phe Ala
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 Ile Ser Gln Ser Ser Thr Gly Thr Val Met Gly Ala Val Ile Met Glu
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 Gly Phe Tyr Val Val Phe Asp Arg Ala Arg Lys Arg Ile Gly Phe Ala
 405 410 415
 Val Ser Ala Cys His Val His Asp Glu Phe Arg Thr Ala Ala Val Glu
 420 425 430
 Gly Pro Phe Val Thr Leu Asp Met Glu Asp Cys Gly Tyr Asn Ile Pro
 435 440 445
 Gln Thr Asp Glu Ser Thr Leu Met Thr Ile Ala Tyr Val Met Ala Ala
 450 455 460
 Ile Cys Ala Leu Phe Met Leu Pro Leu Cys Leu Met Val Cys Gln Trp
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 Ile Ser Leu Leu Lys
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- 4 -

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<211> 476

<212> PRT

<213> Homo sapiens

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 35 40 45
 Glu Glu Pro Glu Glu Pro Gly Arg Arg Gly Ser Phe Val Glu Met Val
 50 55 60
 Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr Val Glu Met Thr
 65 70 75 80
 Val Gly Ser Pro Pro Gln Thr Leu Asn Ile Leu Val Asp Thr Gly Ser
 85 90 95
 Ser Asn Phe Ala Val Gly Ala Ala Pro His Pro Phe Leu His Arg Tyr
 100 105 110
 Tyr Gln Arg Gln Leu Ser Ser Thr Tyr Arg Asp Leu Arg Lys Gly Val
 115 120 125
 Tyr Val Pro Tyr Thr Gln Gly Lys Trp Glu Gly Glu Leu Gly Thr Asp
 130 135 140
 Leu Val Ser Ile Pro His Gly Pro Asn Val Thr Val Arg Ala Asn Ile
 145 150 155 160
 Ala Ala Ile Thr Glu Ser Asp Lys Phe Phe Ile Asn Gly Ser Asn Trp
 165 170 175
 Glu Gly Ile Leu Gly Leu Ala Tyr Ala Glu Ile Ala Arg Leu Cys Gly
 180 185 190
 Ala Gly Phe Pro Leu Asn Gln Ser Glu Val Leu Ala Ser Val Gly Gly
 195 200 205
 Ser Met Ile Ile Gly Gly Ile Asp His Ser Leu Tyr Thr Gly Ser Leu

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210	215	220
Trp Tyr Thr Pro Ile Arg	Arg Glu Trp Tyr Tyr	Glu Val Ile Ile Val
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Arg Val Glu Ile Asn Gly Gln Asp Leu	Lys Met Asp Cys Lys	Glu Tyr
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Asn Tyr Asp Lys Ser Ile Val Asp Ser Gly Thr Thr Asn Leu Arg Leu		
	260	265 270
Pro Lys Lys Val Phe Glu Ala Ala Val Lys Ser Ile Lys Ala Ala Ser		
	275	280 285
Ser Thr Glu Lys Phe Pro Asp Gly Phe Trp Leu Gly Glu Gln Leu Val		
	290	295 300
Cys Trp Gln Ala Gly Thr Thr Pro Trp Asn Ile Phe Pro Val Ile Ser		
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Leu Tyr Leu Met Gly Glu Val Thr Asn Gln Ser Phe Arg Ile Thr Ile		
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Leu Pro Gln Gln Tyr Leu Arg Pro Val Glu Asp Val Ala Thr Ser Gln		
	340	345 350
Asp Asp Cys Tyr Lys Phe Ala Ile Ser Gln Ser Ser Thr Gly Thr Val		
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Met Gly Ala Val Ile Met Glu Gly Phe Tyr Val Val Phe Asp Arg Ala		
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Arg Lys Arg Ile Gly Phe Ala Val Ser Ala Cys His Val His Asp Glu		
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Phe Arg Thr Ala Ala Val Glu Gly Pro Phe Val Thr Leu Asp Met Glu		
	405	410 415
Asp Cys Gly Tyr Asn Ile Pro Gln Thr Asp Glu Ser Thr Leu Met Thr		
	420	425 430
Ile Ala Tyr Val Met Ala Ala Ile Cys Ala Leu Phe Met Leu Pro Leu		
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His Asp Asp Phe Ala Asp Asp Ile Ser Leu Leu Lys		
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<211> 14

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 5

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1 5 10

<210> 6
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<212> PRT
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peptide sequence

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<210> 7
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peptide sequence

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<210> 8
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<212> PRT
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<210> 10
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<220>
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<210> 12
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<212> PRT
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peptide sequence

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<223> Xaa=cysteic acid

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peptide sequence

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peptide sequence

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<223> Xaa= any amino acid

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<210> 16

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peptide sequence

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<223> Xaa= any amino acid

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<222> (4)..(7)

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<210> 17

<211> 8

<212> PRT

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peptide sequence

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<220>
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<210> 19
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<400> 19
Ser Glu Val Asn Leu Asp Ala Glu Phe Arg
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<210> 20
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peptide sequence

<400> 20

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Ser Glu Val Lys Met Asp Ala Glu Phe Arg
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<210> 22
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<210> 23
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peptide sequence

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Trp Lys Lys Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Lys
1 5 10 15

Lys

<210> 24
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peptide sequence

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Ala Asn Leu Ser Thr Phe Ala Gln Pro Arg Arg
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<210> 25

<211> 20

<212> PRT

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<210> 26

<211> 16

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence: synthetic
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<210> 27

<211> 30

<212> PRT

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<220>
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peptide sequence

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peptide sequence

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Leu His Ala Leu Gly Gly Cys
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<400> 31
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<210> 32
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<210> 33
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<210> 34
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<212> PRT
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peptide sequence

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<210> 35
<211> 8
<212> PRT
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peptide sequence

<400> 35
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<210> 36
<211> 8
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peptide sequence

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<210> 37
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peptide sequence

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<210> 38
<211> 7
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<400> 38
Gly Val Leu Leu Ser Arg Lys
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<210> 39
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<212> PRT
<213> Artificial Sequence

<220>
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peptide sequence

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Val Gly Ser Gly Val Leu Leu
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<210> 40

<211> 5

<212> PRT

<213> Artificial Sequence

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peptide sequence

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Val Gly Ser Gly Val
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<210> 41

<211> 12

<212> PRT

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peptide sequence

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<211> 15

<212> PRT

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peptide sequence

<400> 42

Trp Arg Arg Val Glu Ala Leu Tyr Leu Val Glu Gly Glu Arg Lys
1 5 10 15

<210> 43

<211> 14

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

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<210> 44
<211> 4
<212> PRT
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peptide sequence

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<210> 45
<211> 6
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<220>
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peptide sequence

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1 5

<210> 46
<211> 14
<212> PRT
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<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 46
Lys Val Glu Ala Asn Tyr Asp Val Glu Gly Glu Arg Lys Lys
1 5 10

<210> 47
<211> 14
<212> PRT
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<220>
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peptide sequence

<400> 47
Lys Val Glu Ala Asn Leu Ala Val Glu Gly Glu Arg Lys Lys
1 5 10

<210> 48
<211> 14

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<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 48
Lys Val Glu Ala Leu Tyr Ala Val Glu Gly Glu Arg Lys Lys
1 5 10

<210> 49
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
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<220>
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<223> Xaa = E, G, I, D, T, cysteic acid or S

<400> 49
Xaa Ala Asn Tyr Glu Val Glu Phe
1 5

<210> 50
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
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<220>
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<222> (2)
<223> Xaa= A, V, I, S, H, Y, T or F

<400> 50
Glu Xaa Asn Tyr Glu Val Glu Phe
1 5

<210> 51
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
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<222> (3)
<223> Xaa= N, L, K, S, G, T, D, A, Q, or E

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<400> 51
Glu Ala Xaa Tyr Glu Val Glu Phe
1 5

<210> 52
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
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peptide sequence

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<400> 52
Glu Ala Asn Xaa Glu Val Glu Phe
1 5

<210> 53
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<220>
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peptide sequence

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<400> 53
Glu Ala Asn Tyr Xaa Val Glu Phe
1 5

<210> 54
<211> 8
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<220>
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peptide sequence

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<223> Xaa= V, A, N, T, L, F or S

<400> 54
Glu Ala Asn Tyr Glu Xaa Glu Phe
1 5

<210> 55

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<211> 8
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<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<220>
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<222> (7)
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<400> 55
Glu Ala Asn Tyr Glu Val Xaa Phe
1 5

<210> 56
<211> 8
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<220>
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<220>
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<223> Xaa= F, W, G, A, H, P, G, N, S or E

<400> 56
Glu Ala Asn Tyr Glu Val Glu Xaa
1 5

<210> 57
<211> 8
<212> PRT
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<220>
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<400> 57
Xaa Val Leu Leu Ala Ala Gly Trp
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<210> 58
<211> 8
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic

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peptide sequence

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<223> Xaa= A, V, I, S, H, Y, T or F

<400> 58
Gly Xaa Leu Leu Ala Ala Gly Trp
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<210> 59
<211> 8
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peptide sequence

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<400> 59
Gly Val Xaa Leu Ala Ala Gly Trp
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<210> 60
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<400> 60
Gly Val Leu Xaa Ala Ala Gly Trp
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<400> 61
Gly Val Leu Leu Xaa Ala Gly Trp
1 5

<210> 62
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<400> 62
Gly Val Leu Leu Ala Xaa Gly Trp
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<210> 63
<211> 8
<212> PRT
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<220>
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<400> 63
Gly Val Leu Leu Ala Ala Xaa Trp
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<210> 64
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peptide sequence

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<400> 64
Gly Val Leu Leu Ala Ala Gly Xaa
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<210> 65

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<211> 8
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peptide sequence

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<400> 65
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<210> 66
<211> 8
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peptide sequence

<220>
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<400> 66
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<210> 67
<211> 8
<212> PRT
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peptide sequence

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<400> 67
Ile Ile Xaa Met Asp Asn Phe Gly
1 5

<210> 68
<211> 8
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<220>
<223> Description of Artificial Sequence: synthetic

- 23 -

peptide sequence

<220>
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<400> 68
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1 5

<210> 69
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<400> 69
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peptide sequence

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peptide sequence

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<400> 71

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<210> 72

<211> 8

<212> PRT

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peptide sequence

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<400> 72

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1 5

<210> 73

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peptide sequence

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<400> 73

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<210> 74

<211> 10

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<223> Description of Artificial Sequence: synthetic
peptide sequence

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<222> (2)

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<210> 75
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<220>
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<400> 75
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<210> 76
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<400> 76
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<210> 77
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<400> 77
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1 5 10

<210> 78
<211> 10
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<223> Description of Artificial Sequence: synthetic peptide sequence

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<222> (8)

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<210> 79

<211> 9

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<223> Description of Artificial Sequence: synthetic peptide sequence

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<210> 80

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<210> 81

<211> 8

<212> PRT

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<400> 81

Xaa His Gly Phe Gln Leu Xaa His

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<210> 82

<211> 8

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peptide sequence

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<210> 83

<211> 8

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peptide sequence

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<220>

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<222> (7)

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<210> 84

<211> 8

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Thr His Gly Xaa Gln Leu Xaa His
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<210> 85

<211> 8

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1 5

<210> 86

<211> 8

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<223> Description of Artificial Sequence: synthetic peptide sequence

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<223> Xaa= cysteic acid

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<400> 86

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1 5

<210> 87

<211> 8

<212> PRT

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peptide sequence

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Thr His Gly Phe Gln Leu Xaa His
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<210> 88

<211> 8

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peptide sequence

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<400> 88

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<210> 89

<211> 8

<212> PRT

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peptide sequence

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<400> 89

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Xaa Tyr Thr His Ser Phe Ser Pro
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<210> 90

<211> 8

<212> PRT

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<400> 90

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<210> 91

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<212> PRT

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peptide sequence

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<400> 91

Xaa Tyr Xaa His Ser Phe Ser Pro
1 5

<210> 92

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peptide sequence

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Xaa Tyr Thr Xaa Ser Phe Ser Pro
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<210> 93
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peptide sequence

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Xaa Tyr Thr His Ser Xaa Ser Pro
1 5

<210> 95
<211> 8

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<212> PRT
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Xaa Tyr Thr His Ser Phe Xaa Pro
1 5

<210> 96
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<223> Xaa= any amino acid

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Xaa Thr Asp Xaa Gly Ser Xaa Gly
1 5

<210> 98

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peptide sequence

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Ser Xaa Asp Xaa Gly Ser Xaa Gly
1 5

<210> 99

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peptide sequence

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<223> Xaa= any amino acid

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peptide sequence

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peptide sequence

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<222> (5)

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Ser Thr Asp Xaa Xaa Ser Xaa Gly
1 5

<210> 102

<211> 8

<212> PRT

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: synthetic peptide sequence

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<222> (6)

<223> Xaa= V, A, N, T, L, F or S

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Ser Thr Asp Xaa Gly Xaa Xaa Gly
1 5

<210> 103

<211> 8

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<222> (7)

<223> Xaa= E, G, F, H, cysteic acid or S

<400> 103

Ser Thr Asp Xaa Gly Ser Xaa Gly
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<210> 104

<211> 8

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence: synthetic peptide sequence

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<220>
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Xaa Phe Ala Xaa Xaa Xaa Xaa Asn
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<400> 106

Xaa Xaa Ala Xaa Xaa Xaa Xaa Asn

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5

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peptide sequence

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Xaa Phe Xaa Xaa Xaa Xaa Xaa Asn

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<210> 108

<211> 8

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peptide sequence

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<223> Xaa= any amino acid

<400> 108

Xaa Phe Ala Xaa Xaa Xaa Xaa Asn

1

5

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<210> 109
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<220>
<221> SITE
<222> (1)
<223> Xaa= any amino acid

<220>
<221> SITE
<222> (4)
<223> Xaa = any amino acid

<220>
<221> SITE
<222> (5)
<223> Xaa= E, A, D, M, Q, S or G

<220>
<221> SITE
<222> (6)..(7)
<223> Xaa= any amino acid

<400> 109
Xaa Phe Ala Xaa Xaa Xaa Xaa Asn
1 5

<210> 110
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<220>
<221> SITE
<222> (1)
<223> Xaa= any amino acid

<220>
<221> SITE
<222> (4)..(5)
<223> Xaa= any amino acid

<220>
<221> SITE
<222> (6)
<223> Xaa= V, A, N, T, L, F or S

<220>
<221> SITE
<222> (7)
<223> Xaa= any amino acid

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<400> 110
Xaa Phe Ala Xaa Xaa Xaa Xaa Asn
1 5

<210> 111
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<220>
<221> SITE
<222> (1)
<223> Xaa= any amino acid

<220>
<221> SITE
<222> (4)..(6)
<223> Xaa= any amino acid

<220>
<221> SITE
<222> (7)
<223> Xaa= E, G, F, H, cysteic acid or S

<400> 111
Xaa Phe Ala Xaa Xaa Xaa Xaa Asn
1 5

<210> 112
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<220>
<221> SITE
<222> (1)
<223> Xaa= any amino acid

<220>
<221> SITE
<222> (4)..(7)
<223> Xaa= any amino acid

<220>
<221> SITE
<222> (8)
<223> Xaa= F, W, G, A, H, P, G, N or S

<400> 112
Xaa Phe Ala Xaa Xaa Xaa Xaa
1 5

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<210> 113
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 113
Glu Val Asn Leu Asp Ala Glu Phe Arg
1 5

<210> 114
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 114
Asp Tyr Lys Asp Asp Asp Lys
1 5

<210> 115
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 115
Ala Cys Gly Ser Glu Ser Met Asp Ser Gly Ile Ser Leu Asp Asn Lys
1 5 10 15

Trp

<210> 116
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 116
Trp Lys Lys Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Lys
1 5 10 15

Lys

<210> 117

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<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 117
Ala Asn Leu Ser Thr Phe Ala Gln Pro Arg Arg
1 5 10

<210> 118
<211> 22
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 118
Tyr Arg Tyr Gln Ser His Asp Tyr Ala Phe Ser Ser Val Glu Lys Leu
1 5 10 15

Leu His Leu Gly Gly Cys
20

<210> 119
<211> 22
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 119
Tyr Arg Tyr Gln Ser His Asp Tyr Ala Phe Ser Ser Val Glu Lys Leu
1 5 10 15

Leu His Leu Gly Gly Cys
20

<210> 120
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 120
Lys Thr Ile Thr Leu Glu Val Glu Pro Ser
1 5 10

<210> 121
<211> 12

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<212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: synthetic peptide sequence

<220>
 <221> SITE
 <222> (9)
 <223> Xaa= cysteic acid

<400> 121
 Val Glu Ala Leu Tyr Leu Val Cys Xaa Gly Glu Arg
 1 5 10

<210> 122
 <211> 11
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: synthetic peptide sequence

<400> 122
 Val Glu Ala Leu Tyr Leu Val Glu Gly Glu Arg
 1 5 10

<210> 123
 <211> 363
 <212> PRT
 <213> Homo sapiens

<220>
 <223> galactosyltransferase

<400> 123
 Met Ala Ser Lys Ser Trp Leu Asn Phe Leu Thr Phe Leu Cys Gly Ser
 1 5 10 15

Ala Ile Gly Phe Leu Leu Cys Ser Gln Leu Phe Ser Ile Leu Leu Gly
 20 25 30

Glu Lys Val Asp Thr Gln Pro Asn Val Leu His Asn Asp Pro His Ala
 35 40 45

Arg His Ser Asp Asp Asn Gly Gln Asn His Leu Glu Gly Gln Met Asn
 50 55 60

Phe Asn Ala Asp Ser Ser Gln His Lys Asp Glu Asn Thr Asp Ile Ala
 65 70 75 80

Glu Asn Leu Tyr Gln Lys Val Arg Ile Leu Cys Trp Val Met Thr Gly
 85 90 95

Pro Gln Asn Leu Glu Lys Lys Ala Lys His Val Lys Ala Thr Trp Ala
 100 105 110

Gln Arg Cys Asn Lys Val Leu Phe Met Ser Ser Glu Glu Asn Lys Asp
 115 120 125

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Phe Pro Ala Val Gly Leu Lys Thr Lys Glu Gly Arg Asp Gln Leu Tyr
 130 135 140
 Trp Lys Thr Ile Lys Ala Phe Gln Tyr Val His Glu His Tyr Leu Glu
 145 150 155 160
 Asp Ala Asp Trp Phe Leu Lys Ala Asp Asp Thr Tyr Val Ile Leu
 165 170 175
 Asp Asn Leu Arg Trp Leu Leu Ser Lys Tyr Asp Pro Glu Glu Pro Ile
 180 185 190
 Tyr Phe Gly Arg Arg Phe Lys Pro Tyr Val Lys Gln Gly Tyr Met Ser
 195 200 205
 Gly Gly Ala Gly Tyr Val Leu Ser Lys Glu Ala Leu Lys Arg Phe Val
 210 215 220
 Asp Ala Phe Lys Thr Asp Lys Cys Thr His Ser Ser Ser Ile Glu Asp
 225 230 235 240
 Leu Ala Leu Gly Arg Cys Met Glu Ile Met Asn Val Glu Ala Gly Asp
 245 250 255
 Ser Arg Asp Thr Ile Gly Lys Glu Thr Phe His Pro Phe Val Pro Glu
 260 265 270
 His His Leu Ile Lys Gly Tyr Leu Pro Arg Thr Phe Trp Tyr Trp Asn
 275 280 285
 Tyr Asn Tyr Tyr Pro Pro Val Glu Gly Pro Gly Cys Cys Ser Asp Leu
 290 295 300
 Ala Val Ser Phe His Tyr Val Asp Ser Thr Thr Met Tyr Glu Leu Glu
 305 310 315 320
 Tyr Leu Val Tyr His Leu Arg Pro Tyr Gly Tyr Leu Tyr Arg Tyr Gln
 325 330 335
 Pro Thr Leu Pro Glu Arg Ile Leu Lys Glu Ile Ser Gln Ala Asn Lys
 340 345 350
 Asn Glu Asp Thr Lys Val Lys Leu Gly Asn Pro
 355 360

 <210> 124
 <211> 405
 <212> PRT
 <213> Homo sapiens

 <220>
 <223> Homo sapiens sialyltransferase 1

 <400> 124
 Ile His Thr Asn Leu Lys Lys Lys Phe Ser Cys Cys Val Leu Val Phe
 1 5 10 15
 Leu Leu Phe Ala Val Ile Cys Val Trp Lys Glu Lys Lys Lys Gly Ser
 20 25 30
 Tyr Tyr Asp Ser Phe Lys Leu Gln Thr Lys Glu Phe Gln Val Leu Lys

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35					40					45					
Ser	Leu	Gly	Lys	Leu	Ala	Met	Gly	Ser	Asp	Ser	Gln	Ser	Val	Ser	Ser
50						55					60				
Ser	Ser	Thr	Gln	Asp	Pro	His	Arg	Gly	Arg	Gln	Thr	Leu	Gly	Ser	Leu
65					70					75					80
Arg	Gly	Leu	Ala	Lys	Ala	Lys	Pro	Glu	Ala	Ser	Phe	Gln	Val	Trp	Asn
				85					90					95	
Lys	Asp	Ser	Ser	Ser	Lys	Asn	Leu	Ile	Pro	Arg	Leu	Gln	Lys	Ile	Trp
			100					105					110		
Lys	Asn	Tyr	Leu	Ser	Met	Asn	Lys	Tyr	Lys	Val	Ser	Tyr	Lys	Gly	Pro
		115					120					125			
Gly	Pro	Gly	Ile	Lys	Phe	Ser	Ala	Glu	Ala	Leu	Arg	Cys	His	Leu	Arg
	130					135					140				
Asp	His	Val	Asn	Val	Ser	Met	Val	Glu	Val	Thr	Asp	Phe	Pro	Phe	Asn
145						150					155				160
Thr	Ser	Glu	Trp	Glu	Gly	Tyr	Leu	Pro	Lys	Glu	Ser	Ile	Arg	Thr	Lys
				165					170					175	
Ala	Gly	Pro	Trp	Gly	Arg	Cys	Ala	Val	Val	Ser	Ser	Ala	Gly	Ser	Leu
			180					185					190		
Lys	Ser	Ser	Gln	Leu	Gly	Arg	Glu	Ile	Asp	Asp	His	Asp	Ala	Val	Leu
		195					200					205			
Arg	Phe	Asn	Gly	Ala	Pro	Thr	Ala	Asn	Phe	Gln	Gln	Asp	Val	Gly	Thr
	210					215					220				
Lys	Thr	Thr	Ile	Arg	Leu	Met	Asn	Ser	Gln	Leu	Val	Thr	Thr	Glu	Lys
225					230					235					240
Arg	Phe	Leu	Lys	Asp	Ser	Leu	Tyr	Asn	Glu	Gly	Ile	Leu	Ile	Val	Trp
			245						250					255	
Asp	Pro	Ser	Val	Tyr	His	Ser	Asp	Ile	Pro	Lys	Trp	Tyr	Gln	Asn	Pro
			260					265					270		
Asp	Tyr	Asn	Phe	Phe	Asn	Asn	Tyr	Lys	Thr	Tyr	Arg	Lys	Leu	His	Pro
	275						280					285			
Asn	Gln	Pro	Phe	Tyr	Ile	Leu	Lys	Pro	Gln	Met	Pro	Trp	Glu	Leu	Trp
	290					295					300				
Asp	Ile	Leu	Gln	Glu	Ile	Ser	Pro	Glu	Glu	Ile	Gln	Pro	Asn	Pro	Pro
305					310					315					320
Ser	Ser	Gly	Met	Leu	Gly	Ile	Ile	Ile	Met	Met	Thr	Leu	Cys	Asp	Gln
			325						330					335	
Val	Asp	Ile	Tyr	Glu	Phe	Leu	Pro	Ser	Lys	Arg	Lys	Thr	Asp	Val	Cys
			340					345					350		
Tyr	Tyr	Tyr	Gln	Lys	Phe	Phe	Asp	Ser	Ala	Cys	Thr	Met	Gly	Ala	Tyr
	355						360					365			
His	Pro	Leu	Leu	Tyr	Glu	Lys	Asn	Leu	Val	Lys	His	Leu	Asn	Gln	Gly

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370 375 380
 Thr Asp Glu Asp Ile Tyr Leu Leu Gly Lys Ala Thr Leu Pro Gly Phe
 385 390 395 400
 Arg Thr Ile His Cys
 405

 <210> 125
 <211> 518
 <212> PRT
 <213> Homo sapiens

 <220>
 <223> Homo sapiens aspartyl protease 1

 <400> 125
 Met Gly Ala Leu Ala Arg Ala Leu Leu Leu Pro Leu Leu Ala Gln Trp
 1 5 10 15
 Leu Leu Arg Ala Ala Pro Glu Leu Ala Pro Ala Pro Phe Thr Leu Pro
 20 25 30
 Leu Arg Val Ala Ala Ala Thr Asn Arg Val Val Ala Pro Thr Pro Gly
 35 40 45
 Pro Gly Thr Pro Ala Glu Arg His Ala Asp Gly Leu Ala Leu Ala Leu
 50 55 60
 Glu Pro Ala Leu Ala Ser Pro Ala Gly Ala Ala Asn Phe Leu Ala Met
 65 70 75 80
 Val Asp Asn Leu Gln Gly Asp Ser Gly Arg Gly Tyr Tyr Leu Glu Met
 85 90 95
 Leu Ile Gly Thr Pro Pro Gln Lys Leu Gln Ile Leu Val Asp Thr Gly
 100 105 110
 Ser Ser Asn Phe Ala Val Ala Gly Thr Pro His Ser Tyr Ile Asp Thr
 115 120 125
 Tyr Phe Asp Thr Glu Arg Ser Ser Thr Tyr Arg Ser Lys Gly Phe Asp
 130 135 140
 Val Thr Val Lys Tyr Thr Gln Gly Ser Trp Thr Gly Phe Val Gly Glu
 145 150 155 160
 Asp Leu Val Thr Ile Pro Lys Gly Phe Asn Thr Ser Phe Leu Val Asn
 165 170 175
 Ile Ala Thr Ile Phe Glu Ser Glu Asn Phe Phe Leu Pro Gly Ile Lys
 180 185 190
 Trp Asn Gly Ile Leu Gly Leu Ala Tyr Ala Thr Leu Ala Lys Pro Ser
 195 200 205
 Ser Ser Leu Glu Thr Phe Phe Asp Ser Leu Val Thr Gln Ala Asn Ile
 210 215 220
 Pro Asn Val Phe Ser Met Gln Met Cys Gly Ala Gly Leu Pro Val Ala
 225 230 235 240

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Gly Ser Gly Thr Asn Gly Gly Ser Leu Val Leu Gly Gly Ile Glu Pro
 245 250 255
 Ser Leu Tyr Lys Gly Asp Ile Trp Tyr Thr Pro Ile Lys Glu Glu Trp
 260 265 270
 Tyr Tyr Gln Ile Glu Ile Leu Lys Leu Glu Ile Gly Gly Gln Ser Leu
 275 280 285
 Asn Leu Asp Cys Arg Glu Tyr Asn Ala Asp Lys Ala Ile Val Asp Ser
 290 295 300
 Gly Thr Thr Leu Leu Arg Leu Pro Gln Lys Val Phe Asp Ala Val Val
 305 310 315 320
 Glu Ala Val Ala Arg Ala Ser Leu Ile Pro Glu Phe Ser Asp Gly Phe
 325 330 335
 Trp Thr Gly Ser Gln Leu Ala Cys Trp Thr Asn Ser Glu Thr Pro Trp
 340 345 350
 Ser Tyr Phe Pro Lys Ile Ser Ile Tyr Leu Arg Asp Glu Asn Ser Ser
 355 360 365
 Arg Ser Phe Arg Ile Thr Ile Leu Pro Gln Leu Tyr Ile Gln Pro Met
 370 375 380
 Met Gly Ala Gly Leu Asn Tyr Glu Cys Tyr Arg Phe Gly Ile Ser Pro
 385 390 395 400
 Ser Thr Asn Ala Leu Val Ile Gly Ala Thr Val Met Glu Gly Phe Tyr
 405 410 415
 Val Ile Phe Asp Arg Ala Gln Lys Arg Val Gly Phe Ala Ala Ser Pro
 420 425 430
 Cys Ala Glu Ile Ala Gly Ala Ala Val Ser Glu Ile Ser Gly Pro Phe
 435 440 445
 Ser Thr Glu Asp Val Ala Ser Asn Cys Val Pro Ala Gln Ser Leu Ser
 450 455 460
 Glu Pro Ile Leu Trp Ile Val Ser Tyr Ala Leu Met Ser Val Cys Gly
 465 470 475 480
 Ala Ile Leu Leu Val Leu Ile Val Leu Leu Leu Leu Pro Phe Arg Cys
 485 490 495
 Gln Arg Arg Pro Arg Asp Pro Glu Val Val Asn Asp Glu Ser Ser Leu
 500 505 510
 Val Arg His Arg Trp Lys
 515

<210> 126

<211> 255

<212> PRT

<213> Homo sapiens

<220>

<223> Homo sapiens syntaxin 6

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<400> 126

Met Ser Met Glu Asp Pro Phe Phe Val Val Lys Gly Glu Val Gln Lys
 1 5 10 15

Ala Val Asn Thr Ala Gln Gly Leu Phe Gln Arg Trp Thr Glu Leu Leu
 20 25 30

Gln Asp Pro Ser Thr Ala Thr Arg Glu Glu Ile Asp Trp Thr Thr Asn
 35 40 45

Glu Leu Arg Asn Asn Leu Arg Ser Ile Glu Trp Asp Leu Glu Asp Leu
 50 55 60

Asp Glu Thr Ile Ser Ile Val Glu Ala Asn Pro Arg Lys Phe Asn Leu
 65 70 75 80

Asp Ala Thr Glu Leu Ser Ile Arg Lys Ala Phe Ile Thr Ser Thr Arg
 85 90 95

Gln Val Val Arg Asp Met Lys Asp Gln Met Ser Thr Ser Ser Val Gln
 100 105 110

Ala Leu Ala Glu Arg Lys Asn Arg Gln Ala Leu Leu Gly Asp Ser Gly
 115 120 125

Ser Gln Asn Trp Ser Thr Gly Thr Thr Asp Lys Tyr Gly Arg Leu Asp
 130 135 140

Arg Glu Leu Gln Arg Ala Asn Ser His Phe Ile Glu Glu Gln Gln Ala
 145 150 155 160

Gln Gln Gln Leu Ile Val Glu Gln Gln Asp Glu Gln Leu Glu Leu Val
 165 170 175

Ser Gly Ser Ile Gly Val Leu Lys Asn Met Ser Gln Arg Ile Gly Gly
 180 185 190

Glu Leu Glu Glu Gln Ala Val Met Leu Glu Asp Phe Ser His Glu Leu
 195 200 205

Glu Ser Thr Gln Ser Arg Leu Asp Asn Val Met Lys Lys Leu Ala Lys
 210 215 220

Val Ser His Met Thr Ser Asp Arg Arg Gln Trp Cys Ala Ile Ala Ile
 225 230 235 240

Leu Phe Ala Val Leu Leu Val Val Leu Ile Leu Phe Leu Val Leu
 245 250 255

<210> 127

<211> 1728

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: nucleic acid
 encoding recombinant fusion protein

<400> 127

atgctgctgc tgctgctgct gctgggctg aggtacagc tctccctggg catcatccca 60
 gttgaggagg agaaccgga cttctggaac cgcgaggcag ccgaggccct gggcgccgcc 120
 aagaagctgc agcctgcaca gacagccgcc aagaacctca tcattcttct gggcgatggg 180

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atgggggtgt ctacggtgac agctgccagg atcctaaaag ggcagaagaa ggacaaactg 240
gggcctgaga taccctctggc catggaccgc ttcccatatg tggctctgtc caagacatac 300
aatgtagaca aacatgtgcc agacagtgga gccacagcca cggcctacct gtgcgggggtc 360
aagggaact tccagaccat tggcttgagt gcagccgccc gctttaacca gtgcaacacg 420
acacgcgga acgaggtcat ctccgtgatg aatcgggcca agaaagcagg gaagtcagtg 480
ggagtggtaa ccaccacacg agtgcagcac gcctcgccag ccggcaccta cgccacacg 540
gtgaaccgca actggtactc ggacgccgac gtgcctgcct cggcccgcca ggagggtgc 600
caggacatcg ctacgcagct catctccaac atggacattg acgtgatcct aggtggaggc 660
cgaagtaca tgtttcccat gggaaccca gacctgagt acccagatga ctacagccaa 720
ggtgggacca ggctggacgg gaagaatctg gtgcaggaat ggctggcgaa gcgccagggt 780
gcccggatg tgtggaaccg cactgagctc atgcaggctt ccctggaccc gtctgtgacc 840
catctcatgg gtctctttga gcctggagac atgaaatacg agatccaccg agactccaca 900
ctggaccctt ccctgatgga gatgacagag gctgccctgc gcctgctgag caggaaacccc 960
cgcggttct tcctcttctg ggagggtggt cgcacgacc atggtcatca tgaagcagg 1020
gcttaccggg cactgactga gacgatcatg ttgcagcagc ccattgagag ggccggccag 1080
ctcaccagcg aggaggacac gctgagcctc gtactgccc accactccca cgtcttctcc 1140
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gcagtgcgcc tggacgaaga gaccacgcca ggccaggacg tggcggtgtt cgcgcgccc 1380
ccgcaggcgc acctggttca cggcgtgcag gacgagacct tcatagcgca cgtcatggcc 1440
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gacgcgcgcg acccaggtaa ctatgaagtt gaattccgaa gagcactcta cgtagagggt 1560
gaaagaggat tcttctacac tccaaaggca ctctacctg tagagggtga aagaggattc 1620
ttctacacta gtctcatgac catagcctat gtcatggctg ccatctgcgc cctcttcatg 1680
ctgccactct gcctcatggt ggactacaag gatgatgatg acaagtag 1728

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<210> 128

<211> 575

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: recombinant
fusion protein sequence

<400> 128

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Met Leu Leu Leu Leu Leu Leu Gly Leu Arg Leu Gln Leu Ser Leu
  1             5             10             15

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Gly Ile Ile Pro Val Glu Glu Glu Asn Pro Asp Phe Trp Asn Arg Glu
          20             25             30

```

```

Ala Ala Glu Ala Leu Gly Ala Ala Lys Lys Leu Gln Pro Ala Gln Thr
   35             40             45

```

```

Ala Ala Lys Asn Leu Ile Ile Phe Leu Gly Asp Gly Met Gly Val Ser
   50             55             60

```

```

Thr Val Thr Ala Ala Arg Ile Leu Lys Gly Gln Lys Lys Asp Lys Leu
   65             70             75             80

```

```

Gly Pro Glu Ile Pro Leu Ala Met Asp Arg Phe Pro Tyr Val Ala Leu
          85             90             95

```

```

Ser Lys Thr Tyr Asn Val Asp Lys His Val Pro Asp Ser Gly Ala Thr
   100            105            110

```

```

Ala Thr Ala Tyr Leu Cys Gly Val Lys Gly Asn Phe Gln Thr Ile Gly
   115            120            125

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```

Leu Ser Ala Ala Ala Arg Phe Asn Gln Cys Asn Thr Thr Arg Gly Asn
   130            135            140

```


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Glu Val Ile Ser Val Met Asn Arg Ala Lys Lys Ala Gly Lys Ser Val
 145 150 155 160
 Gly Val Val Thr Thr Thr Arg Val Gln His Ala Ser Pro Ala Gly Thr
 165 170 175
 Tyr Ala His Thr Val Asn Arg Asn Trp Tyr Ser Asp Ala Asp Val Pro
 180 185 190
 Ala Ser Ala Arg Gln Glu Gly Cys Gln Asp Ile Ala Thr Gln Leu Ile
 195 200 205
 Ser Asn Met Asp Ile Asp Val Ile Leu Gly Gly Gly Arg Lys Tyr Met
 210 215 220
 Phe Pro Met Gly Thr Pro Asp Pro Glu Tyr Pro Asp Asp Tyr Ser Gln
 225 230 235 240
 Gly Gly Thr Arg Leu Asp Gly Lys Asn Leu Val Gln Glu Trp Leu Ala
 245 250 255
 Lys Arg Gln Gly Ala Arg Tyr Val Trp Asn Arg Thr Glu Leu Met Gln
 260 265 270
 Ala Ser Leu Asp Pro Ser Val Thr His Leu Met Gly Leu Phe Glu Pro
 275 280 285
 Gly Asp Met Lys Tyr Glu Ile His Arg Asp Ser Thr Leu Asp Pro Ser
 290 295 300
 Leu Met Glu Met Thr Glu Ala Ala Leu Arg Leu Leu Ser Arg Asn Pro
 305 310 315 320
 Arg Gly Phe Phe Leu Phe Val Glu Gly Gly Arg Ile Asp His Gly His
 325 330 335
 His Glu Ser Arg Ala Tyr Arg Ala Leu Thr Glu Thr Ile Met Phe Asp
 340 345 350
 Asp Ala Ile Glu Arg Ala Gly Gln Leu Thr Ser Glu Glu Asp Thr Leu
 355 360 365
 Ser Leu Val Thr Ala Asp His Ser His Val Phe Ser Phe Gly Gly Tyr
 370 375 380
 Pro Leu Arg Gly Ser Ser Ile Phe Gly Leu Ala Pro Gly Lys Ala Arg
 385 390 395 400
 Asp Arg Lys Ala Tyr Thr Val Leu Leu Tyr Gly Asn Gly Pro Gly Tyr
 405 410 415
 Val Leu Lys Asp Gly Ala Arg Pro Asp Val Thr Glu Ser Glu Ser Gly
 420 425 430
 Ser Pro Glu Tyr Arg Gln Gln Ser Ala Val Pro Leu Asp Glu Glu Thr
 435 440 445
 His Ala Gly Glu Asp Val Ala Val Phe Ala Arg Gly Pro Gln Ala His
 450 455 460
 Leu Val His Gly Val Gln Glu Gln Thr Phe Ile Ala His Val Met Ala
 465 470 475 480

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Phe	Ala	Ala	Cys	Leu	Glu	Pro	Tyr	Thr	Ala	Cys	Asp	Leu	Ala	Pro	Pro
				485					490					495	
Ala	Gly	Thr	Thr	Asp	Ala	Ala	His	Pro	Gly	Asn	Tyr	Glu	Val	Glu	Pro
			500					505					510		
Arg	Arg	Ala	Leu	Tyr	Val	Glu	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro
		515					520					525			
Lys	Ala	Leu	Tyr	Leu	Val	Glu	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Ser
	530					535					540				
Leu	Met	Thr	Ile	Ala	Tyr	Val	Met	Ala	Ala	Ile	Cys	Ala	Leu	Phe	Met
545					550					555					560
Leu	Pro	Leu	Cys	Leu	Met	Val	Asp	Tyr	Lys	Asp	Asp	Asp	Asp	Lys	
				565					570					575	

<210> 129
 <211> 5
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: synthetic
 peptide sequence

<400> 129
 Lys Met Asp Ala Glu
 1 5

<210> 130
 <211> 5
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: synthetic
 peptide sequence

<400> 130
 Gly Arg Arg Gly Ser
 1 5

<210> 131
 <211> 10
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: synthetic
 peptide sequence

<400> 131
 Val Glu Ala Asn Tyr Glu Val Glu Gly Glu
 1 5 10

<210> 132
 <211> 10

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<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 132
Val Glu Ala Asn Tyr Ala Val Glu Gly Glu
1 5 10

<210> 133
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 133
Lys Thr Ile Asn Leu Glu Val Glu Pro Ser
1 5 10

<210> 134
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<220>
<221> MOD_RES
<222> (5)
<223> Nle

<400> 134
Lys Thr Ile Asn Xaa Glu Val Glu Pro Ser
1 5 10

<210> 135
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (5)
<223> Nle

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 135
Lys Thr Ile Asn Xaa Glu Val Asp Pro Ser

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1	5	10
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<210> 136
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (5)
<223> Nle

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 136
Lys Thr Ile Asn Xaa Asp Val Asp Pro Ser
1 5 10

<210> 137
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 137
Lys Thr Ile Ser Leu Asp Val Glu Pro Ser
1 5 10

<210> 138
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 138
Lys Thr Ile Ser Leu Asp Val Asp Pro Ser
1 5 10

<210> 139
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 139
Lys Met Asp Ala
1

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<210> 140
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 140
Ser Tyr Glu Val
1

<210> 141
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 141
Ser Glu Val Ser Tyr Glu Val Glu Phe Arg
1 5 10

<210> 142
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 142
Asn Leu Asp Ala
1

<210> 143
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 143
Ser Glu Val Ser Tyr Asp Ala Glu Phe Arg
1 5 10

<210> 144
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic

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peptide sequence

<400> 144

Ser	Glu	Val	Ser	Tyr	Glu	Ala	Glu	Phe	Arg
1				5					10

<210> 145

<211> 25

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 145

Thr	Arg	Pro	Gly	Ser	Gly	Leu	Thr	Asn	Ile	Lys	Thr	Glu	Glu	Ile	Ser
1				5				10						15	

Glu	Val	Ser	Tyr	Glu	Val	Glu	Phe	Arg
			20					25

<210> 146

<211> 20

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 146

Gly	Leu	Thr	Asn	Ile	Lys	Thr	Glu	Glu	Ile	Ser	Glu	Val	Ser	Tyr	Glu
1				5					10					15	

Val	Glu	Phe	Arg
			20

<210> 147

<211> 15

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 147

Lys	Thr	Glu	Glu	Ile	Ser	Glu	Val	Ser	Tyr	Glu	Val	Glu	Phe	Arg
1				5					10					15

<210> 148

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

- 55 -

<400> 148

Thr Glu Val Ser Tyr Glu Val Glu Phe Arg
1 5 10

<210> 149

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 149

Ser Glu Val Val Asp Tyr Glu Val Glu Phe Arg
1 5 10

<210> 150

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 150

Thr Glu Val Asp Tyr Glu Val Glu Phe Arg
1 5 10

<210> 151

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 151

Thr Glu Ile Asp Tyr Glu Val Glu Phe Arg
1 5 10

<210> 152

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 152

Ser Glu Ile Ser Tyr Glu Val Glu Phe Arg
1 5 10

<210> 153

<211> 10

- 56 -

<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 153
Ser Glu Ile Asp Tyr Glu Val Glu Phe Arg
1 5 10

<210> 154
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<221> SITE
<222> (11)
<223> Xaa=tryptophan

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 154
Ser Glu Ile Ser Tyr Glu Val Glu Phe Arg Xaa Lys Lys
1 5 10

<210> 155
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<221> SITE
<222> (16)
<223> Xaa=tryptophan

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 155
Lys Thr Glu Glu Ile Ser Glu Ile Ser Tyr Glu Val Glu Phe Arg Xaa
1 5 10 15

Lys Lys

<210> 156
<211> 23
<212> PRT
<213> Artificial Sequence

<220>
<221> SITE
<222> (21)
<223> Xaa=tryptophan

<220>

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<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 156

Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Ile Ser Tyr Glu Val
1 5 10 15

Glu Phe Arg Xaa Lys Lys
20

<210> 157

<211> 28

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

<220>

<221> SITE

<222> (26)

<223> Xaa=tryptophan

<400> 157

Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser
1 5 10 15

Glu Ile Ser Tyr Glu Val Glu Phe Arg Xaa Lys Lys
20 25

<210> 158

<211> 13

<212> PRT

<213> Artificial Sequence

<220>

<221> SITE

<222> (11)

<223> Xaa=tryptophan

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 158

Ser Glu Ile Ser Tyr Glu Val Glu Phe Arg Xaa Lys Lys
1 5 10

<210> 159

<211> 18

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

<220>

<221> SITE

- 58 -

<222> (16)
<223> Xaa=tryptophan

<400> 159
Lys Thr Glu Glu Ile Ser Glu Ile Ser Tyr Glu Val Glu Phe Arg
1 5 10 15

Xaa Lys Lys

<210> 160
<211> 23
<212> PRT
<213> Artificial Sequence

<220>
<221> SITE
<222> (21)
<223> Xaa=tryptophan

<220>
<223> Description of Artificial Sequence: synthetic
peptide

<400> 160
Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Ile Ser Tyr
1 5 10 15

Glu Val Glu Phe Arg Xaa Lys Lys
20

<210> 161
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<221> SITE
<222> (26)
<223> Xaa=tryptophan

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 161
Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile
1 5 10 15

Ser Glu Ile Ser Tyr Glu Val Glu Phe Arg Xaa Lys Lys
20 25

<210> 162
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<221> SITE

- 59 -

<222> (11)

<223> Xaa=oregon green

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 162

Ser Glu Ile Ser Tyr Glu Val Glu Phe Arg Xaa Lys Lys
1 5 10

<210> 163

<211> 18

<212> PRT

<213> Artificial Sequence

<220>

<221> SITE

<222> (16)

<223> Xaa=oregon green

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 163

Lys Thr Glu Glu Ile Ser Glu Ile Ser Tyr Glu Val Glu Phe Arg Xaa
1 5 10 15

Lys Lys

<210> 164

<211> 23

<212> PRT

<213> Artificial Sequence

<220>

<221> SITE

<222> (21)

<223> Xaa=oregon green

<220>

<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 164

Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Ile Ser Tyr Glu
1 5 10 15Val Glu Phe Arg Xaa Lys Lys
20

<210> 165

<211> 28

<212> PRT

<213> Artificial Sequence

<220>

<221> SITE

- 60 -

<222> (26)

<223> Xaa=oregon green

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 165

Thr	Arg	Pro	Gly	Ser	Gly	Leu	Thr	Asn	Ile	Lys	Thr	Glu	Glu	Ile	Ser
1				5				10						15	

Glu	Ile	Ser	Tyr	Glu	Val	Glu	Phe	Arg	Xaa	Lys	Lys
			20					25			

<210> 166

<211> 13

<212> PRT

<213> Artificial Sequence

<220>

<221> SITE

<222> (11)

<223> Xaa=oregon green

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 166

Ser	Glu	Ile	Ser	Tyr	Glu	Val	Glu	Phe	Arg	Xaa	Lys	Lys
1				5				10				

<210> 167

<211> 18

<212> PRT

<213> Artificial Sequence

<220>

<221> SITE

<222> (16)

<223> Xaa=oregon green

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 167

Lys	Thr	Glu	Glu	Ile	Ser	Glu	Ile	Ser	Tyr	Glu	Val	Glu	Phe	Arg
1				5				10					15	

Xaa Lys Lys

<210> 168

<211> 23

<212> PRT

<213> Artificial Sequence

<220>

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<221> SITE
 <222> (21)
 <223> Xaa=oregon green

<220>
 <223> Description of Artificial Sequence: synthetic
 peptide sequence

<400> 168
 Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Ile Ser Tyr
 1 5 10 15
 Glu Val Glu Phe Arg Xaa Lys Lys
 20

<210> 169
 <211> 28
 <212> PRT
 <213> Artificial Sequence

<220>
 <221> SITE
 <222> (26)
 <223> Xaa=oregon green

<220>
 <223> Description of Artificial Sequence: synthetic
 peptide sequence

<400> 169
 Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile
 1 5 10 15
 Ser Glu Ile Ser Tyr Glu Val Glu Phe Arg Xaa Lys Lys
 20 25

<210> 170
 <211> 10
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: synthetic
 peptide sequence

<400> 170
 Ser Glu Val Asn Tyr Glu Val Glu Phe Arg
 1 5 10

<210> 171
 <211> 47
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: synthetic
 primer for site-directed mutagenesis of APP

<400> 171
 gagatctctg aaattagtta tgaagtagaa ttccgacatg actcagg

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<210> 172
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer for site-directed mutagenesis of APP

<400> 172
tgagtcattgt cggaattcta cttcataact aatttcagag atctcctc 48

<210> 173
<211> 47
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer for site-directed mutagenesis of APP

<400> 173
gagatctctg aaagtagtta tgaagtagaa ttccgacatg actcagg 47

<210> 174
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer for site-directed mutagenesis of APP

<400> 174
tgagtcattgt cggaattcta cttcataact actttcagag atctcctc 48

<210> 175
<211> 47
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer for site-directed mutagenesis of APP

<400> 175
gagatctctg aaattagtta tgaagcagaa ttccgacatg actcagg 47

<210> 176
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic primer for site-directed mutagenesis of APP

<400> 176
tgagtcattgt cggaattctg cttcataact aatttcagag atctcctc 48

<210> 177
<211> 5
<212> PRT

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<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 177

Val Ser Tyr Glu Val
1 5

<210> 178

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 178

Val Ser Tyr Asp Ala
1 5

<210> 179

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 179

Ile Ser Tyr Glu Val
1 5

<210> 180

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic peptide sequence

<400> 180

Val Lys Met Asp Ala
1 5

<210> 181

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic primer for generating mutant construct named MBPC125-SYEV

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<400> 181
gacatctctg aagtgagtta ttaggcagaa ttccgacatg actcagg 47

<210> 182
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
primer for generating mutant construct named
MBPC125-SYEV

<400> 182
tgagtcacgt cggaattctg cctaataact cacttcagag atctcctc 48

<210> 183
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 183
Lys Lys Ser Tyr Glu Val
1 5

<210> 184
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 184
Val Glu Ala Asn Tyr Glu Val Glu Gly Glu
1 5 10

<210> 185
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 185
Val Glu Ala Asn Tyr Ala Val Glu Gly Glu
1 5 10

<210> 186
<211> 8
<212> PRT
<213> Artificial Sequence

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<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 186
Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

<210> 187
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 187
Ser Tyr Glu Ala
1

<210> 188
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 188
Ser Tyr Ala Val
1

<210> 189
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
peptide sequence

<400> 189
Val Ser Tyr Glu Ala
1 5

<210> 190
<211> 13
<212> PRT
<213> synthetic peptide sequence

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<400> 190

Ser	Glu	Ile	Ser	Tyr	Glu	Val	Glu	Phe	Arg	Trp	Lys	Lys
1				5					10			

<210> 191

<211> 23

<212> PRT

<213> synthetic peptide sequence

<400> 191

Gly	Leu	Thr	Asn	Ile	Lys	Thr	Glu	Glu	Ile	Ser	Glu	Ile	Ser	Tyr	Glu
1				5					10					15	

Val	Glu	Phe	Arg	Trp	Lys	Lys
			20			

<210> 192

<211> 15

<212> PRT

<213> synthetic peptide sequence

<220>

<221> SITE

<222> (1)..(1)

<223> amino acid at position 1 is biotinylated

<220>

<221> SITE

<222> (14)..(14)

<223> cys at position 14 is derivatized with an oregon green

<400> 192

Lys	Glu	Ile	Ser	Glu	Ile	Ser	Tyr	Glu	Val	Glu	Phe	Arg	Lys	Lys
1				5					10					15

<210> 193

<211> 22

<212> PRT

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<213> synthetic peptide sequence

<220>

<221> SITE

<222> (1)..(1)

<223> amino acid at position 1 is biotinylated

<220>

<221> SITE

<222> (21)..(21)

<223> cys at position 21 is derivatized with an oregon green

<400> 193

Gly	Leu	Thr	Asn	Ile	Lys	Thr	Glu	Glu	Ile	Ser	Glu	Ile	Ser	Tyr	Glu
1				5				10						15	

Val	Glu	Phe	Arg	Lys	Lys
			20		

<210> 194

<211> 6806

<212> DNA

<213> fusion protein comprising a maltose binding protein with 125 amino acids from APP C-terminus.

<400> 194

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gtcaattcag ggtggtgaat gtgaaaccag taacgttata cgatgtcgca gagtatgccg	120
gtgtctctta tcagaccgtt tcccgctgg tgaaccaggc cagccacgtt tctgcgaaaa	180
cgcgggaaaa agtggaagcg gcgatggcgg agctgaatta cattccaac cgctggcac	240
aacaactggc gggcaaacag tcgttgctga ttggcgttgc cacctccagt ctggccctgc	300
acgcgccgtc gcaaattgtc gcggcgatta aatctcgcgc cgatcaactg ggtgccagcg	360
tgggtggtgc gatggtagaa cgaagcggcg tcgaagcctg taaagcggcg gtgcacaatc	420
ttctcgcgca acgcgtcagt gggctgatca ttaactatcc gctggatgac caggatgcca	480
ttgctgtgga agctgcctgc actaatgttc cggcgttatt tcttgatgtc tctgaccaga	540
cacccatcaa cagtattatt ttctcccatg aagacggtac gcgactgggc gtggagcatc	600

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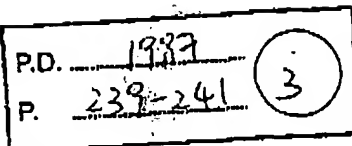
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NOTES & TIPS



An Inverse Mammalian Two-Hybrid System for Beta Secretase and Other Proteases

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It is often difficult to identify and characterize protease cleavage activity without the use of specialized biochemistry or heroic *in vitro* activation of proenzymes. In the case of beta secretase, the N-terminal cleaving enzyme of the beta amyloid peptide, much effort has gone into characterizing candidate enzymes (1). Some of the strategies used in screening for relevant proteases have involved elaborate *in vitro* chromogenic substrates (2) or HPLC separation assays. We describe a simple method based on the two-hybrid system that potentially activates a protease under physiological conditions and allows for the study of known and unknown proteases in mammalian cells.

The yeast two-hybrid system has been shown to be a useful tool in detecting protein-protein interactions (3). The basic strategy is to utilize the properties of the GAL4 protein of *Saccharomyces cerevisiae* for transcriptional activation of genes coding for galactose utilization. The GAL4 protein contains a separate functional domain for specific DNA binding and transcriptional activation. In the two-hybrid system, chimeras of the two functional domains are expressed separately and reconstitution of full functional transcriptional activation can only be achieved when the two domains physically interact.

We describe a strategy based on the two-hybrid system which enables the study of known and unknown proteases through cleavage of a variety of substrates. The focus of our study has been the cleavage of the amyloid precursor protein (APP)¹ by potential beta secretase enzymes. This inverse mammalian two-hybrid system has been studied in transiently transfected 293 cells and utilizes the reporter encoding bacterial chloramphenicol acetyltransferase (CAT) to indirectly detect the affinity of func-

tional proteases for the beta secretase cleaving site. Our results suggest that this system can be used to functionally activate proteases to determine their relevance in protein processing.

Materials and Methods

PCR. Cathepsin G and granzyme B cDNA were generated by PCR amplification. PCR products were amplified using 20 μ M each specific primer, 200 μ M dNTP mix, 1.5 units *Pfu* DNA polymerase, 10 \times *Pfu* buffer (100 mM KCl, 60 mM (NH₄)₂SO₄, 200 mM Tris-HCl, pH 8.0, 20 mM MgCl₂, 1% Triton X-100, 100 μ g/ml BSA), and 250 ng of reverse-transcribed total RNA. Specific primers and cDNA used for each amplification were as follows: cathepsin G (sense primer 5'-ATG CAG CCA CTC CTG CTT CTG CTG-3' and anti-sense primer 5'-TCA CAG GGG GGT CTC CAT CTG ATC-3'), U937 cDNA; granzyme B (sense primer 5'-ATG CAA CCA ATC CTG CTT CTG CTG-3' and anti-sense primer 5'-TTA GTA GCG TTT CAT GGT TTT CTT CTT TAT-3'), human thymus cDNA.

Plasmid constructs. In-frame amyloid precursor protein peptide fusions were constructed by utilizing a *Bgl*II site between serine-236 and arginine-237 of GAL4. Two complementary oligonucleotides encoding the beta-secretase cleavage sequence, within the amyloid precursor protein (coding strand 5'-AAG ACG GAG GAG ATC TCT GAA CTG AAG ATG GAT GCA TTC CGA CAT GAC TCA GGA TAT-3') with compatible *Bgl*II sticky ends were ligated into the *Bgl*II site of pUC119-GAL4. A *Bam*HI/*Xba*I cDNA fragment from pUC119-GAL4/APP was subcloned into the *Bam*HI/*Xba*I site of pRC-CMV (Invitrogen). An expression vector encoding zyme was constructed by ligating *Hind*III/*Xba*I cDNA PCR-amplified products into compatible sites of pRC-CMV. The coding sequences for cathepsin G and granzyme B were PCR amplified and both cathepsin G and granzyme B cDNA were subcloned into the blunted *Bam*HI/*Xba*I site of pRC-CMV.

Transient transfections and CAT assays. Human 293 cells were transfected simultaneously with three plasmids: the modified Gal4/APP construct, the CAT reporter, and the protease-containing pRC-CMV vector at a ratio of 1:1:1. DNA representing the pRC-CMV vector without protease cDNA was substituted when no protease vector DNA was added. Transfections were conducted using a calcium phosphate procedure using 2 μ g each of the effector, reporter, and protease-containing plasmids per 0.3 $\times 10^6$ cells and were assayed for CAT activity as

¹ Abbreviations used: APP, amyloid precursor protein; CAT, chloramphenicol acetyltransferase.

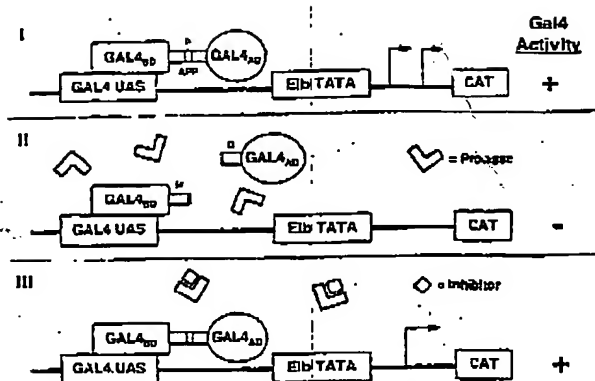


FIG. 1. Model for transcriptional inactivation and reconstitution of GAL4. I. The native GAL4 protein contains the specific substrate and retains transcriptional activation function. II. Expressed protease cleaves the substrate and abolishes GAL4 function. III. Protease inhibitor blocks protease activity and reconstitutes GAL4 transcriptional activation of reporter. Schematic diagram adapted from Refs. 5 and 7.

described by Neumann *et al.* (4). The reporter plasmid containing GAL4 binding sites has been described (5). In cultures containing Pefabloc SC, a serine protease inhibitor, 2 mM was added for 24 h prior to extracting the cells for CAT activity.

One critical parameter in our assay is obtaining high levels of GAL4 induction which subsequently creates a workable window of CAT activity. This was determined empirically by titrating the modified GAL4/APP, reporter, and protease plasmids. Once this has been determined, inducible CAT activity is consistent from one experiment to the next. In addition to determining GAL4 activity, protease expression was determined by Western immunodetection using antibodies specific to each protease.

Western blot analysis. Transfected 293 cells (ATCC No. CRL 1573) were lysed in 200 μ l Tricine/SDS sample buffer (NOVEX) containing 100 mM DTT, 10 μ g/ml leupeptin, 0.2 mM PMSF, and 5 μ g/ml pepstatin A. Samples were heated to 100°C for 5 min, vortexed, microcentrifuged at 14,000g for 5 min, then electrophoresed on a 12.5% Tricine/SDS acrylamide gel. Gels were electroblotted onto PVDF membranes (Bio-Rad) for 1 h at 500 mA in 10 mM Caps, 10% methanol, 0.01% SDS, pH 11.0 buffer. Blots were probed with rabbit polyclonal anti-GAL4 antibody (Santa Cruz Biotechnology Inc.) at 1 μ g/ml. Immunopositive bands were then detected using ECL (Amersham).

Results and Discussion

As in the two-hybrid system, our assay utilizes the physical interaction of the functional domains of GAL4 to create a reporter system for proteases (Fig. 1). The yeast two-hybrid system is based on protein-protein interactions of the DNA binding and transactivating domains of GAL4. Previous studies have shown that the

DNA binding domain specifically binds to a 17-base-pair DNA sequence but does not activate transcription (6). When the carboxy-terminal activation domain becomes proximal to the amino-terminal binding domain, GAL4 directs RNA polymerase II to transcribe the gene downstream of the DNA binding site.

This regulatory mechanism was employed in our protease assay. To determine if a protease contained "beta secretase-like" activity, we inserted a 20-amino-acid cleavage cassette for beta secretase, which is coded within the amyloid precursor protein, into the GAL4 gene of *S. cerevisiae* (7). When this GAL4/APP fusion construct was transiently transfected into 293 cells and indirectly assayed for its functional capabilities through CAT transactivation, this protein retained functional activity. We

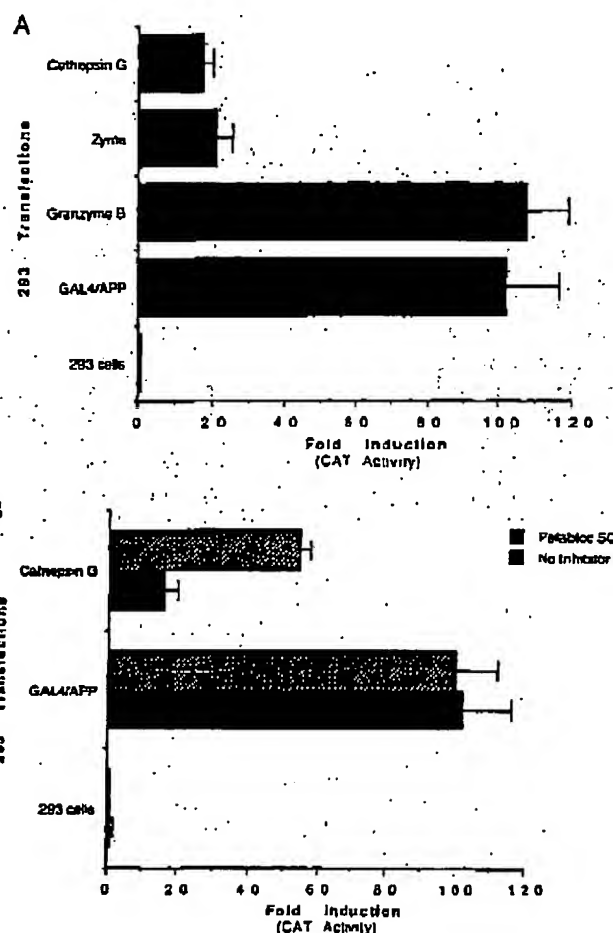


FIG. 2. (A) Histogram of CAT activity in cotransfected 293 cells. Human embryonic kidney cells (293) were cotransfected with the CAT reporter plasmid and the expression plasmid containing cathepsin G, granzyme B, or zyme and the GAL4/APP fusion plasmid. (B) Inhibition of CAT activity in cotransfected cells. Cells cotransfected with cathepsin G, CAT reporter, and the GAL4/APP plasmids were treated with Pefabloc SC as described under Materials and Methods.

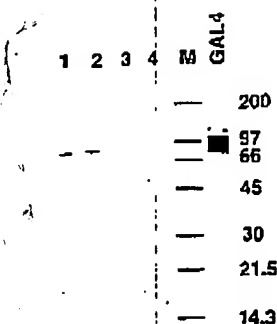


FIG. 3. Western blot analysis using anti-GAL4 antibody. Whole cell extracts from transiently transfected 293 cells were resolved on a denaturing polyacrylamide/SDS gel. Lane 1, GAL4/APP fusion protein. 2, plus granzyme B; 3, plus zyme; 4, plus cathepsin G. M, Amersham rainbow markers; GAL4, purified GAL4 protein.

concluded that 293 cells did not contain enough endogenous beta secretase to limit our assay.

We then sought to determine if our assay would allow for the study of specific proteases that had the potential to process the amyloid precursor protein at the beta secretase site. Several beta secretase candidates have been described (8). Cathepsin G has been found to make the beta secretase cleavage (8) and it is unknown whether granzyme B is active at this site. We were also interested in ascertaining the beta secretase protein processing potential for a novel serine protease, termed zyme, which was cloned in our laboratory (9). After cotransfection of the expression plasmid cDNAs, both zyme and cathepsin G were able to cleave the GAL4/APP cassette and reduce the ability of GAL4 to transactivate the reporter CAT gene (Fig. 2A). In contrast, granzyme B was unable to reduce GAL4 activity showing that the transcription-activation effect is dependent upon specific protease recognition and cleavage. The ability of cathepsin G to recognize the beta amyloid peptide is consistent with its ability to cleave APP *in vitro* (8).

Pefabloc SC is a sulfonil fluoride which irreversibly blocks serine proteases. Because of its low toxicity and stability and its inhibiting activity, we used this inhibitor in our assay to inhibit cathepsin G activity. Reconstitution of 50–60% of control CAT activity was achieved in incubations with Pefabloc SC (Fig. 2B). The remaining decrease in CAT activity may be due to the kinetics of the protease inhibition or from cellular proteases acting at the beta secretase site.

Western blot analysis using anti-GAL4 polyclonal antibodies to the C-terminus of GAL4 demonstrated expression and epitope recognition of the GAL4/APP fusion protein (Fig. 3). Cotransfection with granzyme B showed little or no diminution of expressed GAL4/APP. Consistent with the functional CAT assay data, both zyme and cathepsin G cleave GAL4/APP and reduce the detectable amounts of expressed GAL4/APP fusion.

In conclusion, our assay provides advantages to conventional protease screens. We devised an assay that was designed to activate proteases and assay for enzymatic activity to specific substrates by using a mammalian cell-based system, in conjunction with the described inverse two-hybrid assay. Previous studies in *S. cerevisiae* failed to activate our serine proteases (data not shown). To circumvent this problem, we thought to mimic physiological activation of our proteases by using endogenous cofactors found in human 293 embryonic kidney cells. In addition, our assay provides a rapid and sensitive assay to monitor proteolytic activity and find important therapeutic protease inhibitors. Moreover, we can specifically block those proteases potentially involved in APP processing.

REFERENCES

1. Schenk, D. B., Rydel, R. E., May, P., Little, S., Ponnella, J., Lieberburg, I., and Sinha, S. (1995) *J. Med. Chem.* **38**, 4141–4154.
2. Ladrer, U. S., Wang, G. T., Klein, W. L., Holzman, T. F., and Krafft, G. A. (1994) *J. Protein Chem.* **13**, 357–366.
3. Fields, S., and Song, O. (1989) *Nature* **340**, 245–246.
4. Neumann, J. F., Morency, C., and Russian, K. (1987) *Biotechniques* **5**, 444–447.
5. Sadowski, I., Bell, Brendan, Braid, P., and Hollis, M. (1992) *Gene* **118**, 137–141.
6. Dang, C. V., Barrett, J., Villa-Garcia, M., Resar, L. M. S., Kato, G., and Fearon, E. R. (1991) *Mol. Cell. Biol.* **11**, 954–962.
7. Smith, T. A., and Kohn, B. D. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5159–5162.
8. Brown, A. M., Tammolo, D. M., Spruyt, M. A., Jacobsen, J. S., and Sonnenberg-Reines, J. (1996) *J. Neurochem.* **66**, 2436–2445.
9. Little, S., Johnstone, E., Dixon, E., Norris, F., Buckley, W., Becker, G., Johnson, M., Dohms, J., Wyrick, T., Miller, J., MacKellar, W., Hepburn, D., Corvican, J., McClure, D., Stephenson, D., and Clemens, J. (1995) *J. Cell. Biochem. Suppl.* **21B**, P105.

Quantitative Determination of 2-Monovinyl Protochlorophyll(ide) *b* by Spectrofluorometry¹

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2-Monovinyl (2-MV)³ protochlorophyllide (Pchl_{id}) *b* and its phytol ester [2-MV Pchl(ide) *b*] differ from 2-

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³ Abbreviations used: Pchl_{id}, protochlorophyllide; Pchl_{ide}, Pchl_{id}, Pchl_{ide} phytol ester, or a mixture of both; Chl, chlorophyll; Chl_{id}, chlorophyllide; Chl(ide), Chl, Chl_{id}, or a mixture of both; 2-MV, 2-monovinyl; DV, 2,4-divinyl.

D 10

XP 000616670

Pdr 22-03-'96

P. 7019-7024 = (6)

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Shedding of the Lymphocyte L-Selectin Adhesion Molecule Is Inhibited by a Hydroxamic Acid-based Protease Inhibitor

IDENTIFICATION WITH AN L-SELECTIN-ALKALINE PHOSPHATASE REPORTER*

(Received for publication, December 4, 1995, and in revised form, January 10, 1996)

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Expression of the L-selectin adhesion molecule can be rapidly down-modulated by regulated proteolysis at a membrane-proximal site. The L-selectin secretase has remained undefined, and the secretase activity is resistant to a broad panel of common protease inhibitors. We have developed an L-selectin-alkaline phosphatase reporter, consisting of the ectodomain of human placental alkaline phosphatase fused to the membrane-proximal cleavage, transmembrane, and cytoplasmic domains of L-selectin, to aid in the screening for L-selectin secretase inhibitors. A hydroxamic acid-based metalloprotease inhibitor, KD-IX-73-4, inhibited release of the L-selectin-alkaline phosphatase reporter in a dose-dependent manner. The hydroxamic acid-based peptide was also found to inhibit wild type L-selectin down-regulation from the surfaces of phorbol myristate acetate-activated peripheral blood lymphocytes and phytohemagglutinin-stimulated lymphoblasts. Analysis of the proteolytic cleavage fragments of L-selectin confirmed that KD-IX-73-4 inhibited L-selectin proteolysis. Lymphocyte L-selectin was not down-regulated when co-cultured with formyl-methionylleucylphenylalanine-stimulated neutrophils, suggesting that the putative secretase acts in cis with the membrane-bound L-selectin. These results suggest that the L-selectin secretase activity may involve a cell surface, zinc-dependent metalloprotease, although L-selectin shedding is not affected by EDTA and may be related to the recently described activity involved in processing of membrane-bound TNF- α .

insertion of alanine point mutations at multiple positions surrounding the cleavage site (9, 10). However short deletions in the cleavage region inhibit proteolysis, suggesting that the distance of the cleavage site from the membrane bilayer or the secondary structure of the cleavage domain is important for proteolysis (9, 10).

A number of other leukocyte cell surface markers, including CD43, CD44, CD14, CD16, and CD50, have been shown to be down-regulated upon leukocyte activation (11–17). However the down-regulation of L-selectin shows unusually rapid kinetics (1). Moreover, CD14, CD16, CD43, CD44, and CD50 down-regulation is inhibited by a variety of serine protease inhibitors as well as metalloprotease inhibitors, such as EDTA and 1,10-phenanthroline (11–17). In contrast, L-selectin proteolysis is resistant to these and a broad panel of other common protease inhibitors (11, 15, 18, 19). Thus the class of protease involved in L-selectin shedding has remained unknown.

In this report, we have developed an enzyme reporter construct consisting of the ectodomain of human placental alkaline phosphatase fused to the cleavage, transmembrane, and cytoplasmic domains of L-selectin to screen for novel inhibitors of L-selectin proteolysis. We demonstrate that a hydroxamic acid-based peptide, KD-IX-73-4 inhibits down-regulation of the L-AP¹ reporter. KD-IX-73-4 also inhibits proteolysis of native L-selectin on peripheral blood lymphocytes and PHA-stimulated lymphoblasts. This is the first compound we have found that inhibits L-selectin proteolysis.

MATERIALS AND METHODS

Antibodies—DREG-200 mAb (IgG1) directed against the ectodomain of L-selectin (7) and CA21 mAb (IgG1) and polyclonal antiserum JK584 directed against the cytoplasmic domain of L-selectin (8) have been previously described. Phycoerythrin-conjugated Leu-8 (anti-L-selectin) and isotype control mAb were purchased from Becton-Dickinson (San Jose, CA). Monoclonal antibody 8B6 and polyclonal serum directed against human placental alkaline phosphatase were purchased from Dako (Glostrup, Denmark).

Peripheral Blood Leukocytes—Peripheral blood mononuclear leukocytes were isolated from normal healthy volunteers as described previously (8). PHA lymphoblasts were generated by stimulation of mononuclear leukocytes with 2.5 μ g/ml PHA for 5 days as described previously (8).

L-AP Reporter—The pCMV/SEAP expression vector encoding a soluble form of the human placental alkaline phosphatase gene was purchased from Tropix (Bedford, MA). The L-selectin cDNA in the CDM8 vector was a generous gift of Dr. Brian Seed. The pCMV/SEAP vector was cleaved with *HpaI* and *XbaI* to remove the stop codon and 3'-untranslated sequences of the alkaline phosphatase gene. A fragment

The regulation of adhesion molecule expression and activity is an important facet of modulating cell adhesion and migration. Although a number of strategies to up-regulate receptor activity, both qualitatively and quantitatively, have been elucidated, mechanisms to rapidly down-regulate adhesion have been less well characterized. Expression of the L-selectin (CD62L) adhesion molecule can be rapidly down-regulated from leukocyte cell surfaces upon cell activation (1–7). L-Selectin is cleaved at a membrane-proximal site, which releases a large soluble extracellular fragment (8). Although the cleavage region is well conserved between human, mouse, and rat L-selectin, L-selectin proteolysis is remarkably resistant to the

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¹ The abbreviations used are: L-AP, L-selectin-alkaline phosphatase; mAb, monoclonal antibody; PMA, phorbol myristate acetate; PHA, phytohemagglutinin; TNF, tumor necrosis factor; HPLC, formylmethionyl-leucylphenylalanine; HPLC, high pressure liquid chromatography.

Inhibition of L-Selectin Shedding

encoding the cleavage region (starting with lysine at position 318), transmembrane domain, cytoplasmic domain, and 3'-untranslated regions of L-selectin was amplified by polymerase chain reaction with a specific 5' L-selectin primer (5'-CCAAATTGGACAAAAGTTTC-3') and a 3' CDMB universal primer (5'-CCACAGAAGTAAAGTTTCCTTCA-CAAAG-3'). The 3' end of the amplified fragment was cleaved with XbaI, and the fragment was ligated in-frame with the *HpaI*- and *XbaI*-digested pCMV/SEAP vector, introducing a single serine residue at the boundary of the alkaline phosphatase and L-selectin sequences (see Fig. 1A). The chimeric gene was confirmed by DNA sequencing and expressed transiently in COS cells or stably in L1.2 pre-B cells co-transfected with a puromycin selectable marker. Stable lines were initially selected in 3 μ M puromycin and then analyzed for cell surface expression of human placental alkaline phosphatase.

Alkaline phosphatase activity was initially assessed in adherent COS transfectants by the addition of naphthol phosphate and Fast Red chromagen (Biogenex, San Ramon, CA), which formed a red precipitate around L-AP-transfected cells. The activation-dependent release of the alkaline phosphatase reporter into the supernatant was assessed by harvesting cell-free supernatants from unstimulated or PMA-stimulated L-AP transfectants. L-AP transfectants (2×10^7 cells/ml) were preincubated with compounds for 10 min at room temperature and then activated with 100 ng/ml PMA for 30 min at 37 °C. Cells were pelleted, and 100 μ l of cell-free supernatant was harvested and analyzed for released alkaline phosphatase activity using the Phospha Light Reporter Assay (Tropix, Bedford, MA). Endogenous alkaline phosphatase activity was inactivated by heat treatment for 30 min at 65 °C. The heat-resistant placental alkaline phosphatase was measured by the addition of reaction buffer and CSPD chemiluminescent substrate as per the manufacturer's instructions (Tropix, Bedford, MA). Chemiluminescence was quantitated on a luminometer (Berthold Autolumat LB953). Samples were performed in duplicate or triplicate, as indicated.

Metabolic Labeling.—L-AP transfectants and day 5 PHA lymphoblasts were metabolically labeled with [35 S]methionine and [35 S]cysteine as described previously [8,9].

Immunoprecipitation.—Cell lysates and cell-free supernatants were immunoprecipitated as described previously [8,9]. Immunoprecipitated samples were run on tricine-SDS-polyacrylamide 10–20% gradient gels (Novex). Gels were then fixed in 30% methanol, 10% acetic acid, 1% glycerol, treated with an autoradiography enhancer (Intensify AB, DuPont, NEN), dried in cellophane, and exposed to x-ray film (Kodak X-OMAT) at –70 °C.

Western Blot Analysis.—Western blot analysis with the CA21 mAb was performed as described previously [8], with the exception that the blot was visualized by the addition of ECL chemiluminescent substrate and exposure to film as per the manufacturer's instructions (Amersham Corp.).

Fluorescence-activated Cell Sorter Analysis.—Cells were stained directly with 5 μ l of phycoerythrin-conjugated Leu-8 anti-L-selectin mAb or isotype control mAb (Becton Dickinson) or indirectly with 8B6 anti-alkaline phosphatase mAb or isotype control mAb followed by a second stage phycoerythrin-conjugated goat F(ab')₂ anti-mouse IgG serum in phosphate-buffered saline containing 5% goat serum at 4 °C for 30 min. Cells were then washed twice with goat serum/phosphate-buffered saline fixed in 1% paraformaldehyde/RPMI medium, and 10,000 cells/sample were analyzed by flow cytometry on a FACScan machine (Becton Dickinson).

Synthesis of L-Naphthylalanyl-L-alanine Amide Hydrochloride (L-Nal-L-Ala-NH₂).—Boc-L-Nal-L-Ala-NH₂ was prepared in 90% yield from Boc-Nal-OH and Ala-NH₂ by *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide activation. The HCl salt of the compound was obtained by treatment of the Boc-Nal-L-Ala-NH₂ product with 10 *N* HCl/dioxane, followed by removal of solvent, drying, and crystallization from methanol.

Synthesis of 3-Ethoxycarbonyl-3-(2-methylpropyl)propanoic Acid.—Potassium *t*-butoxide (24.7 g) was dissolved in 200 ml of refluxing *t*-butanol. A mixture of isobutyraldehyde (18.2 ml) and diethyl succinate (41.85 ml) was added to the *t*-butanol solution over 30 min. The reaction mixture was stirred under reflux for 2 h, solvent was removed, and the residue was acidified with 2 *N* HCl. The product was extracted with ethyl ether (Et₂O; 3 \times 200 ml). The ether solution was washed with water, and the product was extracted with 10% Na₂CO₃. The solution was acidified with concentrated HCl, and the product was isolated by extraction with Et₂O. The ether layer was washed with water, dried over sodium sulfate, and evaporated. The remaining oil was dissolved in ethanol and hydrolyzed in the presence of 10% palladium on charcoal. The resultant mixture was filtered through BNSDCCD: <XP_016570A_> (porated to yield 38.04 g of 2-carboethoxy-

carbonyl-4-methyl pentanoic acid as a mixture of isomers in the form of an oil. The isolated monoethyl ester was further purified on silica gel (Kieselgel) 60, 320–400 mesh) by flash chromatography using ethyl acetate/hexane/acetic acid (1:10:0.5, v/v/v). NMR: (300 MHz, CDCl₃): δ (ppm) 0.90 (6H, q, (CH₃)₂CH); 1.20–1.38 (4H, m, CH₂ ester + (CH₃)₂CH); 1.56 (2 H, m, CH₂-CH); 2.45 and 2.72 (2H, dd and q, HOOC-CH₂); 2.84 (1H, m, CH₂-CH-CO); 4.14 (2H, q, CH₂ ester).

Synthesis of 2-Carbo-*t*-butoxycarbonyl-4-methylpentanoic Acid.—To a chilled (–70 °C) solution of 3-(ethoxycarbonyl)-3-(2-methylpropyl)propanoic acid (23 g) in 200 ml of methylene chloride was added isobutylene (200 ml) and concentrated sulfuric acid (4 ml). The reaction mixture was stirred in a glass medium pressure reaction vessel for 4 days at room temperature and then cooled to –70 °C and mixed with 80 ml of saturated NaHCO₃. Standard workup yielded the diester (29.48 g) as an oil. The diester was hydrolyzed with 2 *N* NaOH (40.5 ml) in 200 ml 50% aqueous ethanol for 12 h at room temperature. The ethanol was evaporated, and the remaining oil was diluted with water and extracted with ethyl ether. Following acidification and ether extraction, the obtained monoester (15.6 g) was purified on silica gel by flash chromatography using ethyl acetate/hexane/acetic acid (1:9:0.1, v/v/v). NMR: (300 MHz, CDCl₃): δ (ppm) 0.90 (6H, q, (CH₃)₂CH); 1.27 (1H, m, (CH₃)₂CH); 1.41 (9H, s, (CH₃)₃C); 1.60 (2H, m, (CH₂)₂CHCH₂); 2.34 and 2.55 (1H and 1H, dd and q, CO-CH₂-CH); 2.82 (1H, m, CH-COOH).

Preparation of HONHCOCH₂CH(CH₂CH(CH₃)₂)CO-Nal-Ala-NH₂.—To the chilled (0 °C) solution of above *t*-butyl ester (0.512 g) and *N*-naphthylalanyl-L-alanine amide hydrochloride (0.81 g) in dimethylformamide was added triethylamine (0.42 ml) and then slowly by parts *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (0.48 g) over 15 min. The reaction mixture was stirred for 2 h at 0 °C and left overnight at room temperature. The next day, ethyl acetate (100 ml) was added, and the solution was washed 3 times with 50-ml portions of 1 *N* HCl, NaHCO₃ (sat), brine and then dried over magnesium sulfate. A white solid (0.44 g) obtained after evaporation of solvent was treated with 6*N* HCl/dioxane at room temperature for 1 h to remove the *t*-butyl ester group. Evaporation of dioxane and precipitation with ethyl ether gave a white solid (0.32 g) that was then coupled to *O*-benzyl hydroxylamine using the *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide procedure with triethylamine. Catalytic hydrogenation (10% Pd/C, MeOH, 4 h) furnished the hydroxamic acid product as a mixture of two diastereomers. The isomers were isolated by reversed phase chromatography on a C-18 Dynamax column using an isocratic TFA/AcCN elution mixture. The initial products were lyophilized and characterized as specified below. NMR (Isomer I): (300 MHz, Me₂SO-*d*₆): δ (ppm) 0.64 and 0.73 (6H, 2xd, (CH₃)₂CH); 0.90 (3H, m, (CH₃)₂-CH-CH₂); 1.22 (3H, d, CH₂-CH); 2.84 and 3.00 (1H and 1H, m, CH₂-C₁H₂); 1.84–1.96 (3H, m, CO-CH₂-CH₂); 4.20 (1H, m, CH-CH₂CH₂); 6.984 and 7.21 (1H and 1H, s, CO-NH₂); 7.40–7.90 (8H, m, C₁₀H₇ and CH₂-CH-CO-NH₂); 8.18 (1H, d, CO-NH-CH-CH₂); 8.66 (1H, s, NH-OH). FAB/MS: calc. (M+B) 457.23, found 457. HPLC (Isomer I): Rt = 13.5 min, Hibar C₁₈ (4.7 \times 150 mm), gradient: 30–60% B in 30 min, A: 0.05% TFA; B: 0.05% TFA in AcCN, 1 ml/min. NMR (Isomer II): (300 MHz, Me₂SO-*d*₆): δ (ppm) 0.24 (6H, d, (CH₃)₂CH); 0.38 (4H, m, (CH₃)₂CH and (CH₂)₂CH); 0.70 and 1.08 (1H and 1H, m, (CH₃)₂-CH-CH₂); 1.34 (3H, d, CH₂-CH); 1.92 and 2.02 (1H and 1H, s, CO-CH₂-CH); 2.86 and 3.34 (1H and 1H, m, CH₂-C₁H₂); 2.47 (1H, m, CH₂-CH-CO); 4.23 (1H, m, CH-C₁H₂); 4.55 (1H, m, CHCH₂); 7.02 and 7.15 (1H and 1H, s, CO-NH₂); 7.44–7.78 (7H, m, C₁₀H₇); 8.00 (1H, d, CH₂CO-NH-); 8.42 (1H, d, CO-NH-CH-CH₂); 8.68 (1H, s, NH-OH); 10.50 (1H, s, NH-OH). FAB/MS: calc. (M+B) 457.23, found 457. HPLC (Isomer II): Rt = 15.9 min, Hibar C₁₈ (4.7 \times 150 mm), gradient: 30–60% B in 30 min, A: 0.05% TFA; B: 0.05% TFA in AcCN, 1 ml/min.

RESULTS

An L-Selectin-Alkaline Phosphatase Reporter Construct.—To facilitate the screening of protease inhibitors, we constructed an alkaline phosphatase reporter system to measure proteolysis at the L-selectin cleavage site. Our previous results indicated that the membrane-proximal cleavage domain was necessary to confer proteolysis (9). We fused the 5' sequences encoding the signal sequence and ectodomain of the human alkaline phosphatase gene in-frame with 3' sequences of the L-selectin cDNA encoding the membrane-proximal cleavage domain, the transmembrane domain, and cytoplasmic domain (Fig. 1A). The L-AP chimera was expressed transiently in COS cells (data not shown) and stably in the L1.2 pre-B cell line (Fig. 1, B–D). Cell surface expression was verified by staining with

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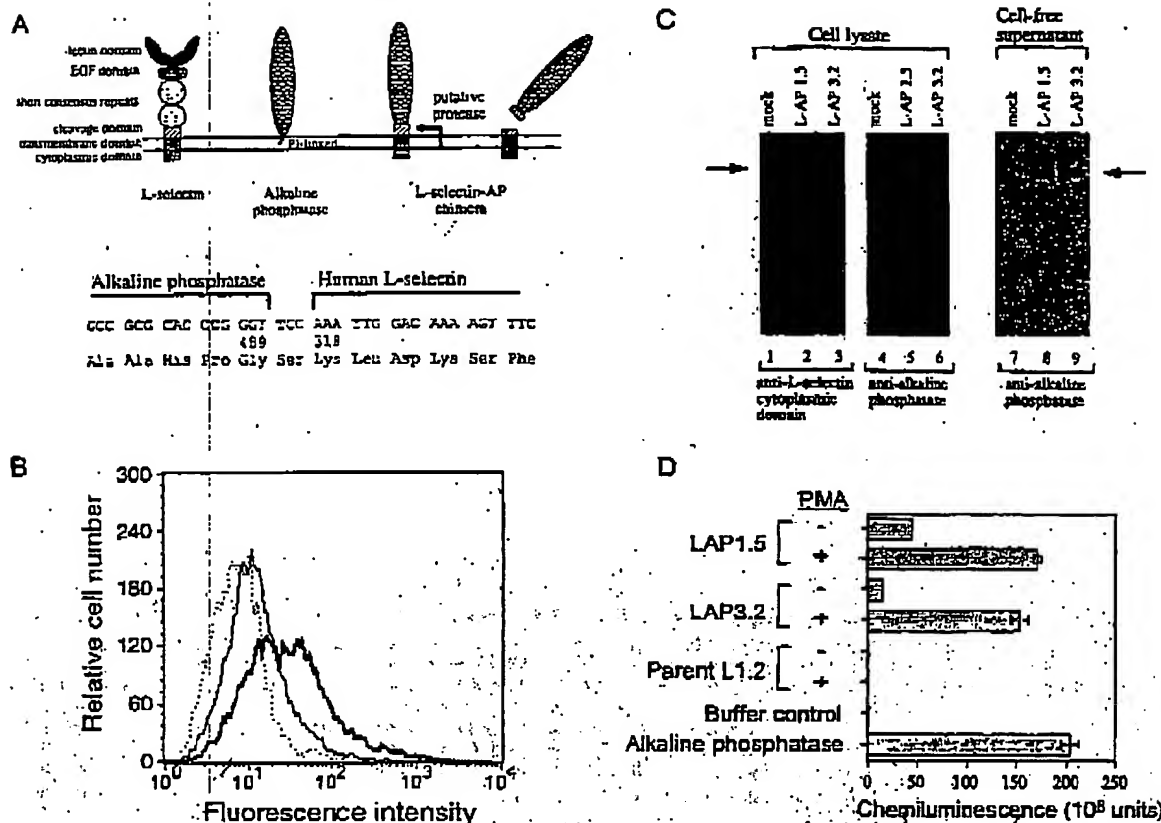


FIG. 1. Construction of an L-AP reporter. **A**, schematic representation of the L-AP reporter generated by fusing the 5' sequences encoding the signal sequence and ectodomain of human placental alkaline phosphatase with the 3' sequences encoding the cleavage, transmembrane, and cytoplasmic domains of L-selectin. Cleavage of the L-AP chimera by a putative membrane-bound protease (right-angle arrow) is illustrated in the upper portion. The nucleotide and amino acid sequences of the junction between amino acid 489 (glycine) of alkaline phosphatase and amino acid 318 (lysine) of the L-selectin are shown in the lower portion. A single serine residue is introduced at the junction. **B**, stable expression of the L-AP reporter in the L1.2 pre-B cell line. Unactivated (thick solid line) or PMA-stimulated (thin solid line) L-AP transfectants were stained with primary mAb directed against human placental alkaline phosphatase and a phycoerythrin-conjugated second stage goat F(ab')₂ anti-mouse IgG and analyzed by flow cytometry. Background staining with an isotype control primary mAb (R6.5) is also shown (dotted line). **C**, immunoprecipitation of the L-AP reporter. Two L-AP-transfected L1.2 cell lines (L-AP1.5 and L-AP3.2) and mock transfectants were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine. Cell lysates were immunoprecipitated with an antibody directed against the ectodomain of alkaline phosphatase (lanes 5 and 6) or with antiserum directed against the cytoplasmic domain of L-selectin (lanes 2 and 3). The membrane bound L-AP chimera is indicated with the left arrow. The released soluble human placental alkaline phosphatase was immunoprecipitated from cell-free supernatants of labeled cells with an anti-human placental alkaline phosphatase antibody (lanes 8 and 9). **D**, assay for released alkaline phosphatase activity. Cell-free supernatants were collected from unactivated and PMA-stimulated parent L1.2 cells and two L-AP-transfected lines, L-AP1.5 and L-AP3.2. An aliquot of the supernatant was mixed with a chemiluminescent substrate for alkaline phosphatase, as described under "Materials and Methods." Emittted light was detected and quantitated on a luminometer. Exogenously added alkaline phosphatase is shown as a positive control. Mean values of triplicate samples (\pm S.D.) for one experiment are shown and are representative of eight similar experiments.

an anti-human placental alkaline phosphatase mAb and analyzed by flow cytometry (Fig. 1B). Specific staining was detected on the L-AP-transfected cells but not on the mock-transfected parent cell line. Expression of the L-AP reporter was down-regulated upon activation with PMA (Fig. 1B) in a manner that paralleled down-regulation of L-selectin on PMA-stimulated L-selectin-transfected L1.2 cells. To further substantiate that the L-AP was expressed and cleaved as expected, the L-AP reporter was analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. L-AP-transfected L1.2 cells were metabolically labeled with [³⁵S]cysteine and [³⁵S]methionine. Cell lysates were immunoprecipitated with anti-alkaline phosphatase antibody or with JK564 antiserum directed against the cytoplasmic domain of L-selectin. Both antibodies immunoprecipitated the full-length membrane-bound chimera of 80 kDa (Fig. 1C, lanes 2, 3, 5, and 6). A soluble 74-kDa fragment of alkaline phosphatase was specifically immunopre-

free supernatants of L-AP-transfected L1.2 cells (Fig. 1C, lanes 8 and 9).

Alkaline phosphatase activity of the L-AP reporter was confirmed by reaction of COS cell transfectants with naphthol phosphate and Fast Red chromagen, a substrate that reacts with alkaline phosphatase and resulted in the formation of a red precipitate around L-AP-transfected cells but not around mock-transfected cells (data not shown). The L-AP transfectants showed a low background of alkaline phosphatase release in the absence of PMA activation. However upon stimulation with PMA, a 5-8-fold increase in alkaline phosphatase activity was released into the supernatant. No alkaline phosphatase activity was detectable from the parent L1.2 cells in the presence or absence of PMA (Fig. 1D).

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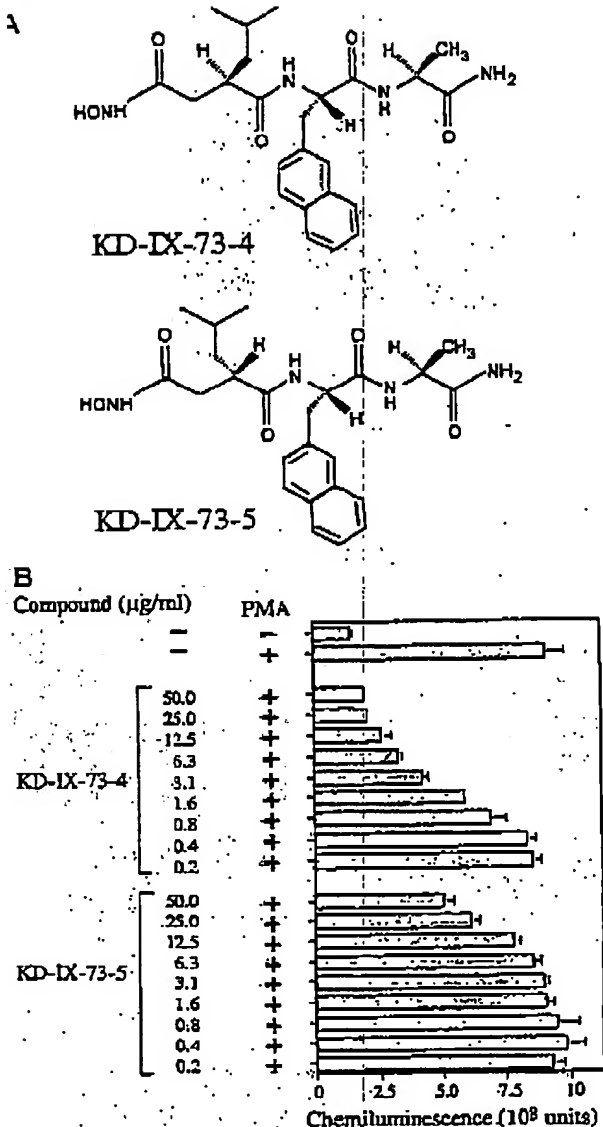


FIG. 2. Release of the L-AP reporter is inhibited by hydroxamic acid-based peptides. **A**, structure of KD-IX-73-4 (*N*-[(2-(hydroxymethyl)-4-methylpentanoyl)-L-3-(2'-naphthyl)alanine]-L-alanine amide) and its diastereomer KD-IX-73-5 (*N*-[(2-(hydroxymethyl)-4-methylpentanoyl)-L-3-(2'-naphthyl)alanine]-L-alanine amide). The diastereomers were separated by reverse phase HPLC. **B**, inhibition of L-AP reporter release. L-AP transfectants were preincubated with various concentrations of compounds as indicated for 10 min and then activated with 100 ng/ml PMA for 30 min at 37 °C. Cell-free supernatants were harvested and assayed for released alkaline phosphatase activity as described in the legend to Fig. 1. Mean values (\pm S.D.) of duplicate samples for one experiment are shown and are representative of four similar experiments.

Proteolysis of the L-AP Reporter Is Inhibited by Peptide Hydroxamic Acids—We tested a series of peptide hydroxamic acids, which were originally synthesized as inhibitors of zinc-dependent matrix metalloproteases. KD-IX-73-4 and KD-IX-73-5 are two diastereomers (Fig. 2A). KD-IX-73-4 is a potent collagenase and gelatinase inhibitor, whereas the diastereomer KD-IX-73-5 is much less active against matrix metalloproteases (Fig. 2B). KD-IX-73-4 inhibited L-AP proteolysis in a dose-dependent manner (Fig. 2B) and reduced L-AP release to near

background levels at 25–50 μg/ml. The IC₅₀ of inhibition with KD-IX-73-4 was approximately 4.5 μM. In contrast, the diastereomer KD-IX-73-5 was at least 20–25-fold less potent than KD-IX-73-4. Alkaline phosphatase is a metal-dependent enzyme whose activity is inhibited by EDTA. Indeed 5 mM EDTA inhibits the L-AP reporter assay (Table I). To determine if KD-IX-73-4 specifically inhibits the putative secretase or non-specifically inhibits the activity of the alkaline phosphatase reporter, we tested the effects of EDTA and KD-IX-73-4 on exogenous soluble human placental alkaline phosphatase. EDTA completely inhibited alkaline phosphatase activity, as expected (Table I). However KD-IX-73-4 had no significant effect on exogenous alkaline phosphatase activity, indicating that it did not nonspecifically inhibit activity of the reporter enzyme.

The Hydroxamic Acid-based Peptide Inhibits Lymphocyte L-Selectin Shedding—We next tested whether the peptide hydroxamic acid compounds would inhibit down-regulation of wild type L-selectin from peripheral blood lymphocytes. Lymphocytes were pretreated with KD-IX-73-4 or the diastereomer KD-IX-73-5 for 10 min and then activated with 100 ng/ml PMA. KD-IX-73-4 inhibited L-selectin down-regulation in a dose-dependent manner in response to PMA stimulation, as judged by cell surface expression of L-selectin (Fig. 3). The IC₅₀ of inhibition was approximately 8 μM. Pretreatment of lymphocytes with 50 μg/ml KD-IX-73-4 followed by washing the compound out prior to activation had minimal effects on subsequent down-regulation of L-selectin (Fig. 3). These results suggest that the protease is protected from the action of the compound until cell activation or that the compound readily dissociates from the protease. The less active diastereomer KD-IX-73-5 had partial activity at 50 μg/ml and no appreciable activity at low concentrations. In contrast, 5 mM EDTA had no effect on L-selectin shedding (Table I) as described previously (11, 19).

To confirm that the KD-IX-73-4 inhibited L-selectin proteolysis, cleavage products of L-selectin were analyzed by immunoprecipitation from metabolically labeled PMA-stimulated lymphoblasts. We have previously shown that the 74-kDa membrane form of L-selectin is cleaved to release a 68-kDa soluble fragment and a 6-kDa transmembrane fragment (8). Immunoprecipitation from cell lysates with JK564 serum directed against the cytoplasmic domain of L-selectin revealed that treatment with KD-IX-73-4 prevented the disappearance of the full-length 74 kDa L-selectin and inhibited appearance of the 6-kDa transmembrane cleavage product from PHA lymphoblasts stimulated with PMA (Fig. 4, lane 3 versus lane 2). Similarly immunoprecipitation from cell-free supernatants with JK923 serum directed against the ectodomain of L-selectin revealed that treatment with KD-IX-73-4 inhibited the release of the 68-kDa soluble form of L-selectin (Fig. 4, lane 6 versus lane 5). The small amount of cleavage products observed in the KD-IX-73-4-treated lymphoblasts may be due in part to spontaneous proteolysis that occurred during the metabolic pulse and chase periods, when the KD-IX-73-4 compound was not present.

The L-Selectin Protease Activity Acts in cis with Membrane-bound L-Selectin—We next examined whether the protease activity is likely to be a soluble secreted enzyme or a membrane-bound enzyme. We incubated neutrophils and lymphocytes together at a ratio of 7:1 and stimulated the cells with 100 ng/ml PMA or 10⁻⁷ M FMLP. Both lymphocytes and neutrophils responded to PMA by down-regulating L-selectin expression (Fig. 5). However FMLP, which stimulates neutrophils but not lymphocytes, caused L-selectin down-regulation selectively on the neutrophil population. These results indicate that the pu-

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TABLE I

Inhibition of the L-AP reporter assay, exogenous alkaline phosphatase activity, and leukocyte L-selectin shedding by metalloprotease inhibitors

Inhibitor ^a	Percent inhibition = S.D. (n)		
	L-AP reporter assay ^b	Exogenous alkaline phosphatase ^c	L-Selectin shedding ^d
EDTA	85.8 ± 3.5 (2)	88.2 ± 0.8 (2)	2.7 ± 4.7 (3)
KD-IX-73-4	83.8 ± 0.9 (2)	7.6 ± 5.5 (2)	84.7 ± 10.9 (3)

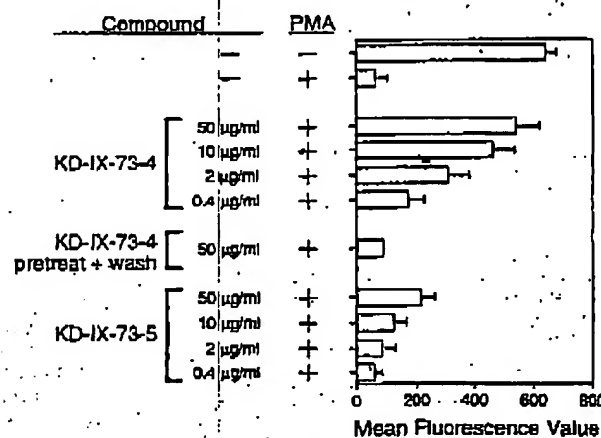
^a EDTA and KD-IX-73-4 were tested at 5 mM and 50 µg/ml, respectively, in all assays.^b L-AP transfected cells were pretreated with compounds and activated with 100 ng/ml PMA, and supernatant was assayed for released alkaline phosphatase activity, as described under "Materials and Methods."^c Purified human placental alkaline phosphatase was treated with compounds and assayed for residual alkaline phosphatase activity.^d Freshly isolated peripheral blood leukocytes were treated with compounds and activated with 100 ng/ml PMA. Cells were stained for residual L-selectin expression with a phycoerythrin-conjugated anti-L-selectin mAb and analyzed by flow cytometry.

Fig. 3. Hydroxamic acid compounds inhibit L-selectin down-regulation on lymphocytes. Peripheral blood lymphocytes were treated with KD-IX-73-4 or its diastereomer KD-IX-73-5, as indicated, and activated with 100 ng/ml PMA for 30 min at 37 °C as indicated. Some cells, as indicated, were pretreated with 50 µg/ml KD-IX-73-4 and then washed twice to remove compound prior to the addition of PMA. The cells were stained with phycoerythrin-conjugated Leu-8 anti-L-selectin mAb or with phycoerythrin-conjugated control mAb (not shown), and 10,000 events per sample were analyzed by quantitative flow cytometry. Mean fluorescence values (\pm S.D.) from three experiments are plotted.

tative secretase acts in cis with membrane-bound L-selectin and suggest that it is not a freely soluble activity.

DISCUSSION

We have found that L-selectin proteolysis is inhibited by a hydroxamic acid-based compound, KD-IX-73-4. Mechanistically the hydroxamic acid moiety functions to coordinate zinc metal, and thus the hydroxamic acid-based peptides are potent inhibitors of zinc-dependent matrix metalloproteases such as collagenase and gelatinase (20). Our results suggest that the L-selectin secretase may involve a metal-dependent protease, possibly a member of the metalloprotease family. However, previous results have shown that L-selectin down-regulation is resistant to several general metalloprotease inhibitors, such as 1,10-phenanthroline (11) and EDTA (11, 15, 18, 19). In contrast, matrix metalloproteases are inhibited by both EDTA and 1,10-phenanthroline. Thus the exact class of protease cannot be ascertained at this time. The observation that EDTA but not KD-IX-73-4 inhibits the metal-dependent activity of exogenous alkaline phosphatase suggests that KD-IX-73-4 is not acting nonspecifically as a general metal chelator.

Although the KD-IX-73-4 compound is a potent inhibitor of collagenase and gelatinase, it is unlikely that the natural L-selectin secretase is the common soluble form of collagenase.

The IC₅₀ of KD-IX-73-4 against L-selectin secretase is about 100 µg/ml, less than that for collagenase (20). Moreover two



Fig. 4. Hydroxamic acids inhibit proteolysis of L-selectin. Day 6 PHA lymphoblasts were metabolically pulse-labeled with [³⁵S]methionine and [³⁵S]cysteine for 30 min and chased for 30 min in the absence of inhibitors. Labeled cells were then preincubated with KD-IX-73-4 compound for 10 min as indicated and then activated with 100 ng/ml PMA for 30 min as indicated. Cell lysates were immunoprecipitated with JK564 serum directed against the cytoplasmic domain of L-selectin. Cell-free supernatants were immunoprecipitated with JK923 serum directed against the ectodomain of L-selectin. Samples were subjected to SDS-polyacrylamide gelelectrophoresis and autoradiography with fluorography.

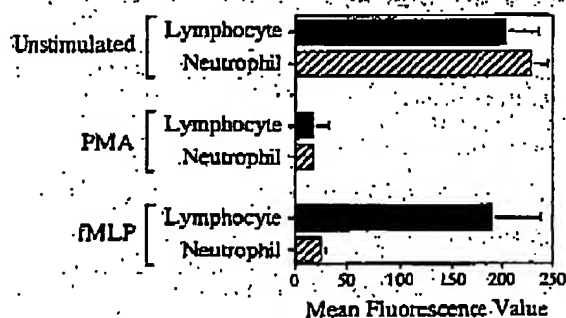


Fig. 5. L-Selectin is cleaved in cis. Neutrophils and lymphocytes were co-cultured at a ratio of 7:1 and a total cell concentration of 1×10^6 cells/ml. The cell mix was left unstimulated or was stimulated with 50 ng/ml PMA or 10^{-7} M fMLP, as indicated, for 30 min at 37 °C. The cells were stained with phycoerythrin-conjugated Leu-8 anti-L-selectin mAb or with phycoerythrin-conjugated control mAb (not shown) and analyzed by quantitative flow cytometry. L-Selectin expression on neutrophil versus lymphocyte populations was determined by appropriate forward- and side-scatter analysis gates. Mean fluorescent values (\pm S.D.) for two separate experiments are plotted.

specific inhibitors of collagenase and gelatinase were previously found to be inactive in inhibiting L-selectin proteolysis (11). Our indirect evidence suggests that the L-selectin secretase is a membrane-bound activity. Neutrophils and lymphocytes both respond to PMA activation and down-regulate L-selectin from their cell surfaces. However, fMLP stimulates only neutrophils to down-regulate L-selectin, even if lymphocytes are co-incubated with fMLP-stimulated neutrophils at

high cell densities. These results suggest that the secretase acts in cis with the membrane-bound L-selectin. A membrane-bound protease constrained in the same two-dimensional membrane plane as L-selectin would effectively increase the local substrate concentration for the putative secretase and may explain in part the rapid kinetics of L-selectin proteolysis. Indeed the relatively poor potency of the KD-IX-73-4 compound may be due in part to the rapid kinetics of the secretase activity following cell activation.

Recently three groups have shown that hydroxamic acid-based peptides can inhibit processing of a membrane-bound form of TNF- α to a soluble form (21–23). KD-IX-73-4 is structurally identical to compound one in the study by Mohler *et al.* (21), and its potency against TNF- α processing is similar to what we found for L-selectin proteolysis. A similar compound, TNF- α protease inhibitor, has also been found to inhibit release of the TNF receptor (24). Notably TNF- α processing, in contrast to L-selectin proteolysis, is inhibited by EDTA (21). Moreover TNF- α is a type II protein, whereas L-selectin is a type I protein. L-Selectin and TNF- α are among an emerging class of diverse proteins, such as transforming growth factor- α (25–27), β -amyloid precursor protein (28, 29), IL-6 receptor (30, 31), TNF receptor (32), and angiotensin-converting enzyme (33, 34), which are regulated by rapid and inducible cleavage from the cell surface (35). Significantly, the native protease involved in any of these secretase activities has not been described. The availability of a protease inhibitor will aid in the purification and identification of the natural L-selectin secretase.

Finally, the hydroxamic acid-based inhibitors will allow us to probe the contribution of L-selectin shedding to L-selectin function. Recently we have demonstrated that hydroxamic acid-based inhibitors significantly reduce neutrophil rolling velocity on L-selectin ligands in an *in vitro* model of hydrodynamic flow.² These results suggest that L-selectin proteolysis occurs during L-selectin-mediated rolling interactions and that shedding contributes significantly to the velocity of rolling. The multistep adhesion cascade model predicts a well orchestrated interplay of selectin, chemokine, and integrin functions. It is possible that L-selectin shedding facilitates this transition and that inhibition of L-selectin shedding might impact downstream events, including transendothelial migration.

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² B. Walcheck, J. Kahn, C. Feehan, R. Batagery, K. Darlak, A. F. Spetola, and T. K. Kishimoto, submitted for publication.

REFERENCES

- Kishimoto, T. K., Jutila, M. A., Berg, E. L., and Butcher, E. C. (1989) *Science* 245, 1238–1241.
- Jutila, M. A., Rott, L., Berg, E. L., and Butcher, E. C. (1989) *J. Immunol.* 143, 3318–3324.
- Griffin, J. D., Spertini, O., Ernst, T. J., Belvin, M. P., Levine, H. B., Kanakura, Y., and Tedder, T. F. (1990) *J. Immunol.* 145, 576–584.
- Berg, M., and James, S. P. (1990) *Blood* 76, 2381–2388.
- Jung, T. M., and Dailey, M. O. (1990) *J. Immunol.* 144, 3180–3188.
- Spertini, O., Freedman, A. S., Belvin, M. P., Penta, A. C., Griffin, J. D., and Tedder, T. F. (1991) *Leukemia* 5, 300–308.
- Kishimoto, T. K., Jutila, M. A., and Butcher, E. C. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 2244–2248.
- Kahn, J., Ingraham, R. H., Shirley, F., Migaki, G. I., and Kishimoto, T. K. (1994) *J. Cell Biol.* 125, 481–470.
- Migaki, G. I., Kahn, J., and Kishimoto, T. K. (1995) *J. Exp. Med.* 182, 549–557.
- Chen, A., Engel, P., and Tedder, T. F. (1995) *J. Exp. Med.* 182, 519–530.
- Basil, V., and Strominger, J. L. (1994) *J. Immunol.* 152, 1314–1322.
- Basil, V., and Strominger, J. L. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 3792–3796.
- Basil, V., and Horejzky, V. (1992) *J. Immunol.* 149, 747–753.
- Basil, V., and Strominger, J. L. (1991) *J. Immunol.* 147, 1567–1574.
- Campanero, M. R., Pulido, R., Alonso, J. L., Pineda, J. P., Pimentel-Munoz, F. X., Fresno, M., and Sanchez-Madrid, F. (1991) *Eur. J. Immunol.* 21, 3045–3048.
- Rieu, P., Porteu, F., Bessou, G., Lasseve, P., and Halbwachs-Mecarelli, L. (1992) *Eur. J. Immunol.* 22, 3021–3026.
- Del Pozo, M. A., Pulido, R., Muñoz, C., Alvarez, V., Kumbria, A., Campanero, M. R., and Sanchez-Madrid, F. (1994) *Eur. J. Immunol.* 24, 2586–2594.
- Shipp, M. A., Stefano, G. B., Switzer, S. N., Griffin, J. D., and Reinherz, E. L. (1991) *Blood* 78, 1824–1841.
- Kishimoto, T. K., Kahn, J., Migaki, G., Mainolfi, E., Shirley, F., Ingraham, R., and Rothlein, R. (1995) *Agents Actions* 47, (suppl.) 121–124.
- Spetola, A. F., Darlak, K., Pegoraro, S., Nijhawan, K., Anzillo, M., Lankiewicz, L., and Gray, R. D. (1992) in *Peptides: Chemistry and Biology* (Smith, J. A., and Rivier, J. E., eds) pp. 820–821, ESCOM, Leiden.
- Mohler, K. M., Sleath, P. R., Fitzner, J. N., Carretti, D. P., Alderson, M., Karwar, S. S., Torrance, D. S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., Kronhelm, S. R., Peterson, M., Garhart, M., Kozlosky, C. J., March, C. J., and Black, R. A. (1994) *Nature* 370, 218–220.
- Gearing, A. J. H., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., Gordon, J. L., Leber, T. M., Mangan, M., Müller, K., Noyes, P., Owen, K., Patel, S., Thomas, W., Wells, G., Wook, L. M., and Woolley, K. (1994) *Nature* 370, 555–557.
- McGeachan, G. M., Becherer, J. D., Best, R. C., Jr., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Fardon, P., Karp, S., Kline, S., McElroy, A. B., Nichols, J., Pryzwansky, K. M., Schoenen, F., Schot, L., Truesdale, A., Verghese, M., Warner, J., and Ways, J. P. (1994) *Nature* 370, 558–561.
- Crowe, P. D., Walzer, B. N., Mohler, K. M., Otten-Evans, C., Black, R. A., and Ware, C. F. (1995) *J. Exp. Med.* 181, 1205–1210.
- Arribes, J., and Massagué, J. (1995) *J. Cell Biol.* 128, 433–441.
- Bosenberg, M. W., Pandiella, A., and Massagué, J. (1993) *J. Cell Biol.* 122, 65–101.
- Pandiella, A., and Massagué, J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 1726–1730.
- Sahasrabudhe, S. R., Spruyt, M. A., Muenkel, E. A., Bhume, A. J., Vitek, M. P., and Jacobsen, J. S. (1992) *J. Biol. Chem.* 267, 25603–25608.
- Shioda, S. S. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 6075–6079.
- Mullberg, J., Oberthur, W., Lottspeich, F., Mehl, E., Dietrich, E., Graeve, L., Heinrich, P. C., and Rose-John, S. (1994) *J. Immunol.* 152, 4856–4868.
- Mullberg, J., Schrodink, K., Spony, T., Gunther, M., Graeve, L., Buse, G., Mackiewicz, A., Heinrich, P. C., and Rose-John, S. (1993) *Eur. J. Immunol.* 23, 473–480.
- Porteu, F., and Nathan, C. (1990) *J. Exp. Med.* 172, 589–607.
- Ehlers, M. R. W., Chen, Y.-N. P., and Riordan, J. F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 1009–1013.
- Ramchandran, R., Sen, G. C., Misra, K., and Sen, I. (1994) *J. Biol. Chem.* 269, 2125–2130.
- Ehlers, M. R. W., and Riordan, J. F. (1991) *Biochemistry* 30, 10066–10074.

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A Sensitive and Quantitative Assay for Measuring Cleavage of Presenilin Substrates*

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The presenilin (PS) proteins are components of the γ -secretase activity, which is central in the pathogenesis of Alzheimer's disease. Here we present a novel cell-based reporter gene assay for the quantification of PS-controlled γ -secretase cleavage of the Alzheimer amyloid precursor protein (APP). We show that this assay offers several advantages, including increased sensitivity and specificity, improved quantification of cleavage, and simultaneous detection of all γ -secretase cleavages in APP. Furthermore, the APP assay can be used in parallel with a similar assay that records γ -secretase cleavage of a Notch receptor. The use of these assays to analyze the effects of two known γ -secretase inhibitors and postulated PS active site mutants on APP and Notch processing demonstrated that inhibitors and mutants that differently affect Notch and APP cleavage can be identified rapidly. The possibility in using these assays for high throughput screening of candidate γ -secretase inhibitors for APP and Notch in parallel opens up new vistas to systematically search for novel inhibitors that selectively block APP cleavage while not affecting Notch signaling.

PS¹ proteins and APP are frequently mutated in familial Alzheimer's disease (AD), and PS controls the γ -secretase-mediated processing of APP. The level of γ -secretase activity controls the amount of amyloid β -peptide (A β) formation from the APP intermediate fragment C99 (see Fig. 1A). Familial AD-associated mutations in PS lead to a specific increase in production of the more fibril-prone A β 42 variant (1), a peptide species linked to the early pathological changes seen in AD (2,

3). It is therefore of interest to develop pharmaceutical approaches that reduce the level of γ -secretase activity acting on APP. However, a complicating factor for this approach is that PS also controls cleavage of Notch receptors (4), which are critical for many functions during development and in the adult organism. γ -Secretase inhibitors aimed at reducing A β formation might thus also affect Notch cleavage and have unwanted side effects. Therefore, high throughput screening assays that record γ -secretase cleavage of both APP and Notch would be useful for the identification of novel APP-specific γ -secretase inhibitors. In this study we present a novel cell-based reporter gene assay for monitoring γ -secretase cleavage on APP.

EXPERIMENTAL PROCEDURES

DNA Constructs—Silent mutations were first incorporated in a pcDNA3-C99 construct to create an AclI site immediately 3' of the nucleotides encoding the transmembrane-spanning region of C99. A cDNA encoding the DNA binding/transactivation domains Gal4/VP16 (GVP) was subsequently amplified by PCR and cloned into the AclI site of C99 generating the C99-GVP construct. Both PS1 and PS2 cDNAs were obtained in the pcDNA3 backbone. All PS1 mutants were generated by PCR using *Pfu* Turbo polymerase according to the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA). The PS1 D257A/D385A double mutant construct was generated out of the PS1 D257A- and PS1 D385A-encoding plasmids by a subcloning approach. All constructs were verified by sequencing using the ABI377 automated sequencer (PerkinElmer Life Sciences). The construct encoding Notch Δ E-GVP and the UAS-responsive reporter gene construct MH100 and the CMV- β -gal plasmid have been described previously.²

Cell Transfection Experiments and Reporter Gene Assay—HEK293 cells and blastocyst-derived embryonic stem cells lacking PS1 and PS2 expression, ED8 cells, were cultured as described previously.² All transfections with ED8 cells were carried out in 24-well tissue culture plates (Costar), and transfection of 293 cells was carried out in 10-cm cell culture dishes (Costar). For each well of the 24-well tissue culture dish, 200 ng of MH100, 50 ng of CMV- β -gal plasmid, 100 ng of Notch Δ E-GVP or C99-GVP plasmid, and either 100 ng of PS1, PS2, or empty pcDNA3 plasmids were mixed with PLUS reagent and LipofectAMINE according to the recommendations of the manufacturer (Invitrogen). The cells were harvested and analyzed for reporter gene activity 48 h post-transfection. For the 10-cm dishes, 2 μ g of MH100, 400 ng of CMV- β -gal, and either 2 μ g of C99-GVP or Notch Δ E-GVP were mixed and used to transfect the 293 cells according to the LipofectAMINE PLUS protocol. The transfected 293 cells were split and seeded into 24-well plates, and γ -secretase inhibitors were added. Twelve hours postdrug addition, the cells were harvested and analyzed as described previously.²

RESULTS AND DISCUSSION

In this report we describe a new sensitive and quantitative assay for γ -secretase-mediated cleavage of APP. To generate a reporter APP protein, from which γ -secretase cleavage can be recorded, we incorporated a Gal4 DNA-binding/VP16 transactivation (GVP) domain into the C99 form of APP (Fig. 1A), which is an immediate substrate for γ -secretase *in vivo* (5). Cleavage of the resulting hybrid protein, hereafter referred to as C99-GVP, liberates the C-terminal region, including the GVP moiety, which is translocated to the nucleus due to the nuclear localization signals in GVP. In the nucleus, GVP specifically signals through a UAS-luciferase reporter gene by virtue of the strong transactivation domain and specific binding to a UAS promoter (Fig. 1A). The assay has several attractive features. First, it should be specific for γ -secretase cleavage

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‡ The abbreviations used are: PS, presenilin; AD, Alzheimer's disease; APP, amyloid precursor protein; A β , amyloid β -peptide; GVP, Gal4/VP16; UAS, upstream activation sequence.

² Y. Taniguchi, H. Karlström, J. Lundkvist, T. Mizutani, A. Otaka, M. Vestling, A. Bernstein, D. Dunviel, U. Landahl, and T. Honjo, submitted.

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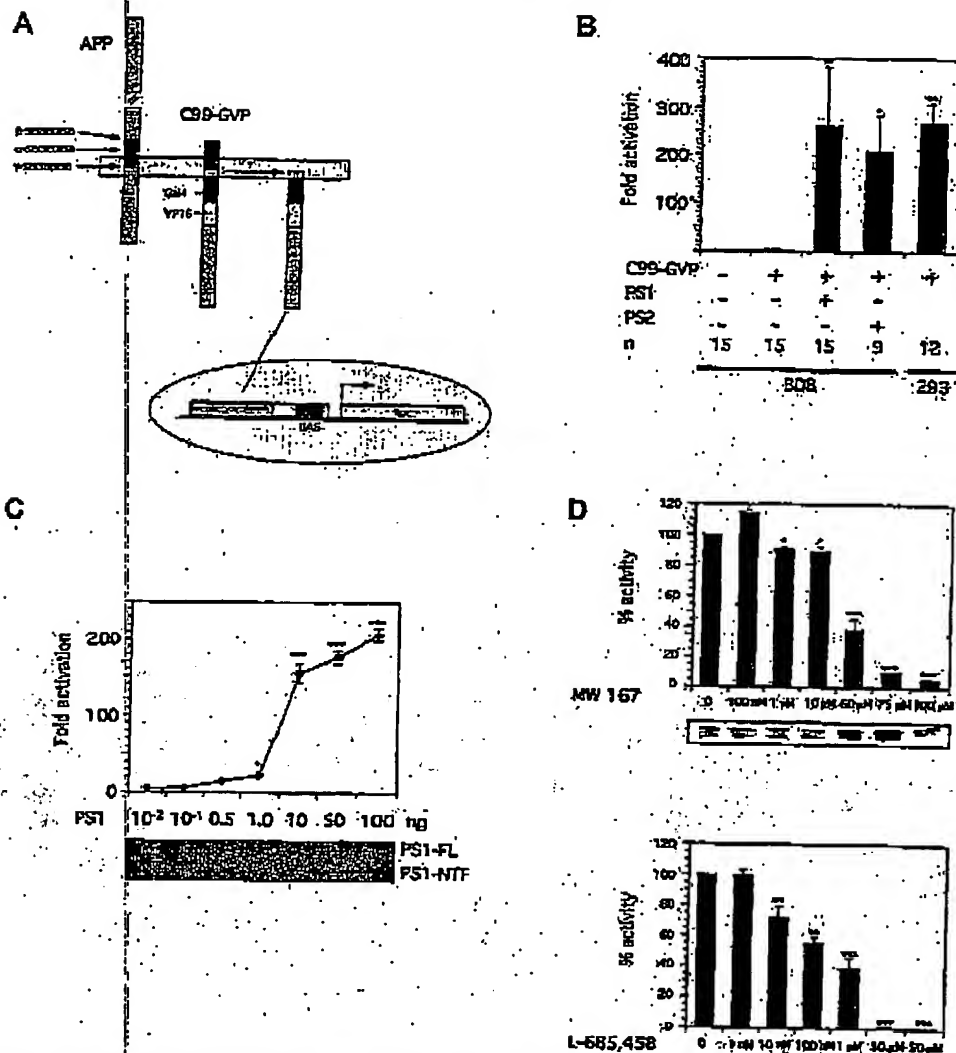
Novel Assay for γ -Secretase Activity

FIG. 1. Proteolytic processing of C99-GVP is dependent on PS and sensitive to γ -secretase inhibitors. *A*, schematic view of the processing events of APP and the γ -secretase assay. The three cleavage sites in APP (α , β , and γ) are depicted. The A β domain is shown in red. C99 is a direct substrate for γ -secretase cleavage. Following cleavage at the γ -secretase site, the intracellular domain containing the GVP domain translocates to the nucleus and initiates luciferase expression from the UAS promoter. *B*, C99-GVP is only a substrate for γ -secretase in the presence of PS. γ -Secretase-mediated cleavage of C99-GVP was analyzed in transiently transfected cells deficient for PS1 and PS2 (BD8 cells, bars 1–4) or in cells containing both PS1 and PS2 genes (HEK293 cells, bar 5). No cleavage is observed in BD8 cells transfected with C99-GVP in the absence of PS (bar 2), while substantial processing is recorded from the UAS-luc reporter gene after co-transfection of PS1 cDNA (bar 3) and PS2 cDNA (bar 4). Similar levels of cleavage were observed in the HEK293 cells transfected with C99-GVP-encoding cDNA (bar 5). The experiments were performed in triplicates and repeated at least three times. Data are presented as fold activation of mock-transfected cells and show mean \pm S.E. *** , $p < 0.01$ and * , $p < 0.05$ versus mock. *C*, the processing of C99-GVP is dependent on but saturable to the amount of PS expressed. Processing of C99-GVP in BD8 cells was analyzed in the presence of different amounts of transfected PS1-encoding cDNA. Note that above 10 ng of transfected PS1-encoding cDNA only a very minor increase in processing activity on the C99-GVP substrate was observed. Data are presented as fold activation of mock-transfected cells and show mean \pm S.E. of two independent experiments with triplicates of each treatment. *** , $p < 0.001$ and * , $p < 0.05$ versus 0 ng of PS1 cDNA. Below is shown a Western blot examining the PS1 expression levels in the transfected BD8 cells using the antibody Ab14 (1:1000). *D*, γ -secretase inhibitors block the processing of C99-GVP in a dose-dependent manner. The effect of two γ -secretase inhibitors, MW167 and L-685,438, on the processing of C99-GVP was analyzed in transiently transfected HEK293 cells. Both inhibitors caused a dose-dependent decrease in γ -secretase activity as measured by luciferase activity. The experiment was repeated at least three times with duplicates of each treatment. Data are presented as percent activity where 100% activity corresponds to the reporter gene activity obtained in the absence of γ -secretase inhibitors. The graph shows mean \pm S.E. Parts of the extracts were analyzed by Western blot for C99-GVP expression using the 369 antibody (1:1000). Note that the slightly higher expression of C99-GVP in the presence of 50–76 μ M MW167 does not result in an elevated reporter gene activity. This indicates the specificity of the assay for recording γ -secretase activity. *** , $p < 0.001$; ** , $p < 0.01$; and * , $p < 0.05$ versus C99-GVP with Me₂SO. *n*, number of experiments; FL, full length; NTF, N-terminal fragment.

since the GVP moiety is inserted only 18–15 amino acid residues C-terminal of the γ -secretase cleavage sites, thus minimizing unspecific cleavage events. Second, it should be very sensitive since VP16 is a strong transactivation domain on the

UAS promoter. Third, it will record the total γ -secretase cleavage activity, i.e. γ -cleavage generating both A β 40, the more abundant A β variant (6), and the pathogenic A β 42 species as well as minor cleavage products generated by processing at

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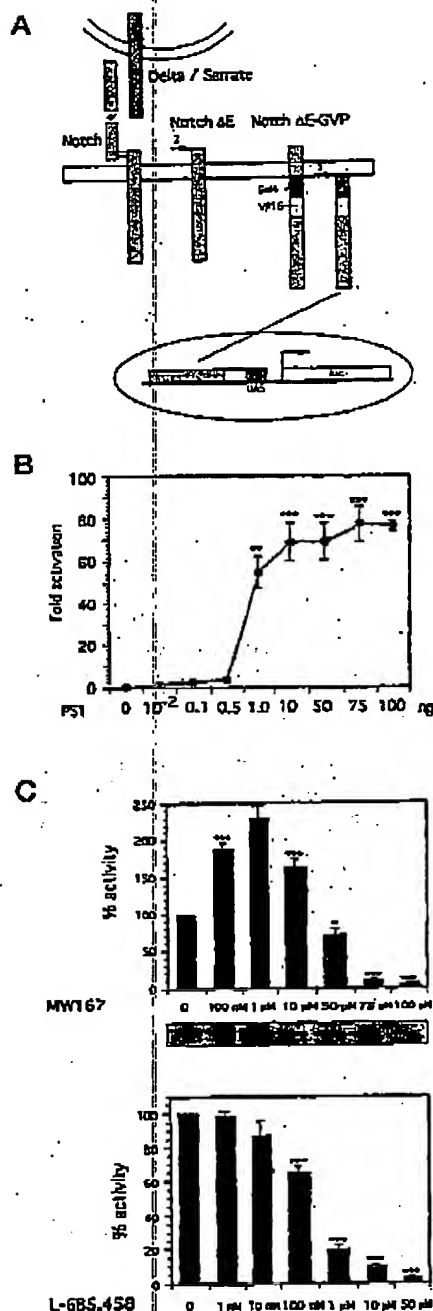


Fig. 2. Processing of Notch Δ E-GVP is PS-dependent and susceptible to γ -secretase inhibitors. **A**, overview of the ligand-induced processing events of the Notch receptor and the γ -secretase assay. Notch Δ E is a truncated form of a Notch receptor, which is cleaved at site 2 and site 3 in a ligand-independent constitutive manner. Following cleavage at the γ -secretase site (site 3) the intracellular domain containing the GFP domain translocates to the nucleus and initiates luciferase expression from the UAS promoter. **B**, PS proteins are required for Notch Δ E-GVP processing. Cleavage of Notch Δ E-GVP in BD8 cells was analyzed in the presence of different amounts of transfected PS1-encoding cDNA. As for C99-GVP (Fig. 1C), only a very minor increase in γ -secretase-mediated cleavage of the Notch Δ E-GVP substrate was observed above 10 ng of transfected PS1-encoding cDNA. Data are presented as fold activation of mock-transfected cells and show mean \pm

adjacent residues will be measured simultaneously. This is a marked improvement over current γ -secretase assays that rely primarily on analyzing secreted A β species with specific antibodies while excluding the large, and structurally distinct (7), intracellular pool of A β . Moreover, the C99-GVP construct harbors the native APP cytoplasmic tail, ensuring correct intracellular trafficking by the sorting signals contained within the tail (8, 9). Finally, the assay records cleavage only from the C99-GVP protein and is thus insensitive to the levels of endogenous γ -secretase substrates in the cell.

We first examined the specificity of the C99-GVP assay by recording γ -cleavage in cells expressing different combinations of PS genes. After transfection of C99-GVP into HEK293 cells, which express endogenous levels of wild-type PS1 and PS2, a strong activation of the UAS-luciferase gene was observed (Fig. 1B). In contrast, transfection of C99-GVP into BD8 cells, which lack PS1 and PS2 expression, did not activate the reporter gene (Fig. 1B), indicating that cleavage is completely dependent on the presence of PS proteins. To test the assay for PS1 and PS2 individually, we transfected expression constructs of PS1 and PS2 into the BD8 cells and measured the cleavage of C99-GVP. This resulted in strong reporter gene activation in both cases, demonstrating that both PS1 and PS2 are capable of mediating γ -cleavage of C99-GVP (Fig. 1B). These results are in agreement with recent reports from *Drosophila* in which a similar construct was successfully used for genetic analysis of PS-controlled γ -cleavage (10).

To address whether the assay was quantitative, we transfected different amounts of PS1 into BD8 cells. The level of reporter gene activation increased with the amount of PS expressed, primarily in the range from 10 pg to 10 ng of added PS1 plasmid DNA (Fig. 1C). Interestingly, above 10 ng, only a minor increase in cleavage activity was observed. This may suggest that the production of the activated form of PS required for cleavage is limiting (i.e. by adding too much PS, the proteolytic machinery generating the N- and C-terminal PS fragments is saturated) or, alternatively, that a component other than PS in the active γ -secretase complex is the rate-limiting determinant of cleavage (11). We also tested whether the assay responded quantitatively to the addition of two known γ -secretase inhibitors, MW167 (12) and L-685,458 (13). Addition of both MW167 and L-685,458 resulted in a dose-dependent decrease in γ -secretase activity (Fig. 1D).

As discussed above, γ -secretase inhibitors that do not discriminate between APP and Notch cleavage may have dangerous side effects if used in a therapeutic setting. Both APP and Notch are cleaved at defined sites in the intramembrane region, and thus it should be possible to use a very similar GVP-based assay to monitor γ -secretase-mediated cleavage (site 3 cleavage) of Notch (Fig. 2A). We have recently shown that a truncated Notch receptor that acts in a ligand-independent constitutive manner (14) and harbors the GVP moiety inserted immediately C-terminal of the site 3 cleavage site (Notch Δ E-GVP) is cleaved specifically in the presence of PS in

S.E. of two independent experiments with triplicates of each treatment. ***, $p < 0.001$ and **, $p < 0.01$ versus 0 ng PS1 cDNA. **C**, γ -secretase inhibitors inhibit processing of Notch Δ E-GVP. As for C99-GVP, both MW167 and L-685,458 caused a dose-dependent decrease in γ -secretase activity. The experiment was repeated at least three times with duplicates of each treatment. Data are presented as percent activity where 100% activity corresponds to the reporter gene activity obtained in the absence of γ -secretase inhibitors. The graph shows mean \pm S.E. of three independent experiments with triplicates of each treatment. ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$ versus Notch Δ E-GVP with Me₂SO. Protein levels of Notch Δ E-GVP in the presence of different concentrations of MW167 were assessed by Western blotting using the monoclonal c-Myc antibody 9E10 (1:200). luc, luciferase.

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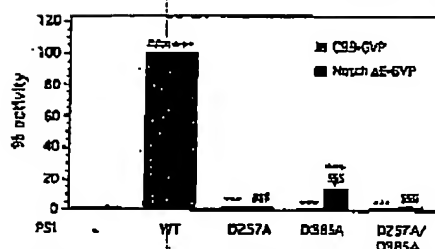
Novel Assay for γ -Secretase Activity

FIG. 3. PS1 active site mutants differentially affect γ -secretase-mediated cleavage of C99-GVP and Notch Δ E-GVP. cDNAs encoding PS1 D257A, D385A, or D257A/D385A were transfected into ED8 cells together with either C99-GVP or Notch Δ E-GVP. γ -Secretase-mediated processing was recorded as luciferase activity where the activity obtained in the presence of wild-type PS1 was set to 100%. Note that the expression of none of the mutants resulted in any detectable processing from either the C99-GVP or the Notch Δ E-GVP construct, except for the D385A mutant, which rescued cleavage from the Notch Δ E-GVP construct by 15%. [The graph shows mean \pm S.E. of three independent experiments with triplicates of each transfectant. ###, $p < 0.001$ versus C99-GVP without PS1 cDNA. +++, $p < 0.001$ versus Notch Δ E-GVP without PS1 cDNA. **, $p < 0.001$ versus C99-GVP + PS1 wild-type cDNA. §§, $p < 0.001$ versus Notch Δ E-GVP + PS1 wild-type cDNA. WT, wild type.]

cultured cells.² We show here that Notch Δ E-GVP, like C99-GVP, can respond in a graded fashion to varying amounts of PS (Fig. 2B) and γ -secretase inhibitors (Fig. 2C). As in the case for C99-GVP, addition of PS1 above 10 ng does not significantly increase Notch cleavage efficiency. In addition, preliminary experiments suggest that expression of increasing amounts of Notch Δ E results in a dose-dependent attenuation of the processing of C99-GVP (data not shown). These data further demonstrate the similarities in processing between APP and Notch.

Both γ -secretase inhibitors displayed similar potency in inhibiting Notch Δ E-GVP and C99-GVP processing. The IC_{50} value for MW167 was calculated to be $\sim 50 \mu M$, which is slightly higher than the previously reported value of $20 \mu M$ (20). This discrepancy in IC_{50} values probably reflects the different methodologies used in the two investigations. L-685,458 inhibited C99-GVP and Notch Δ E-GVP processing with an IC_{50} value of 200 nM. The IC_{50} for C99-GVP was comparable to the IC_{50} reported for L-685,458 in intact cells (48–775 nM depending on the cell line used and whether secreted A β 40 or A β 42 was measured) (13). Thus, the similarity of the data obtained in this study and other reports confirm the validity of the C99-GVP reporter gene assay to address γ -secretase activity on APP.

To learn whether the assay was capable of recording differences between Notch and APP processing, we used artificial missense mutations in PS that affect two aspartate residues at positions 257 and 385 thought to be involved in the PS active site that have been shown to differentially alter APP and Notch processing (17). We generated single or double aspartate to alanine mutants at positions 257 and 385 in PS1 and tested the effects in the C99 and Notch GVP assays in the PS-deficient BD8 cells (Fig. 3). Transient expression of the D257A, D385A, and D257A/D385A mutants into BD8 cells resulted in no detectable signaling from the C99-GVP reporter construct. In contrast, the D385A mutant rescued 15% of the signaling for Notch Δ E-GVP, while the other two mutants did not activate the reporter gene. This finding is interesting in light of previous data. Our data on APP processing support the original finding by Wolfe and co-workers (18) that the aspartate residues are critical in PS-mediated cleavage of APP but disagree with data from Haass *et al.* (17). The D385A mutant has in all previous reports been shown to inhibit Notch processing in a

potent way (17, 18). The residual cleavage activity of the D385A PS1 mutant in our assay was therefore unexpected but may reflect the sensitivity of the GVP-based assay and that it was conducted in a PS-null background, avoiding possible interference of wild-type, endogenously expressed PS. Moreover, recent data have emerged that challenge the view that PS proteins are direct executors of the γ -secretase cleavage (19).² Irrespective of the finer details, our data show that the GVP-based assays for APP and Notch are able to identify situations in which APP and Notch processing differ.

In conclusion, we provide a novel assay to monitor γ -secretase processing of APP and show that it can be used together with a similar assay for Notch processing. The APP assay is highly sensitive and quantitative and can simultaneously record all γ -secretase-mediated cleavages. The fact that the GVP-based assays for APP and Notch are based on transfected cells also render these assays suitable for large scale testing of multiple compounds for γ -secretase inhibition in an unbiased high throughput fashion. In principle, it should be possible to screen several thousands of compounds for their effect on γ -secretase activity in a few days. The observation that these assays can score differences in processing between APP and Notch makes the assays very attractive as tools to identify novel γ -secretase modulators that differentially affect APP and Notch processing.

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REFERENCES

- Hardy, J. (1997) *Trends Neurosci.* 20, 184–188
- Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N., and Ihara, Y. (1994) *Neuron* 13, 45–53
- Naslund, J., Haroutunian, V., Mohr, R. C., Davis, K. L., Davies, P., Greengard, P., and Blalock, J. D. (2000) *J. Am. Med. Assoc.* 283, 1571–1577
- De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessens, K., Mumm, J. S., Schreuer, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Guste, A., and Kopan, R. (1999) *Nature* 398, 518–523
- Herrmann, A., Smeekle, L., Annaert, W., Collen, D., Schreuer, E., and De Strooper, B. (2000) *Nat. Cell Biol.* 2, 461–463
- Schreuer, E., Eickholt, C., Jensen, M., Song, X., Chiriac, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Vektem, M., Pickford, E., Pookaj, P., Schenk, G., Tanzi, R., Wasen, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1995) *Nat. Med.* 1, 864–870
- Skovronsky, D. M., Dome, R. W., and Lee, V. M.-Y. (1998) *J. Cell Biol.* 141, 1031–1039
- Lei, A., Sisodia, S. S., and Treier, J. S. (1993) *J. Biol. Chem.* 268, 3565–3573
- Haass, C., Koo, E. H., Capell, A., Teplow, D. B., and Selkoe, D. J. (1996) *J. Cell Biol.* 133, 537–547
- Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525
- Yu, G., Chen, F., Nishimura, M., Salner, H., Tandon, A., Kawarai, T., Arisaka, S., Supala, A., Song, Y.-Q., Rogava, E., Holmes, E., Zhang, D. M., Milman, P., Fraser, P. E., Haass, C., and St. George-Hyslop, P. (2000) *J. Biol. Chem.* 275, 27848–27853
- Wolfe, M. S., Xia, W., Moore, C. L., Leatherwood, D. D., Ostaszewski, B., Rahmad, T., Donker, I. O., and Selkoe, D. J. (1999) *Biochemistry* 38, 4720–4727
- Shearman, M. S., Behr, D., Clarke, E. F., Lewis, H. D., Harrison, T., Hunt, P., Nadin, A., Smith, A. I., Stevenage, G., and Castro, J. L. (2000) *Biochemistry* 39, 8698–8704
- Mumm, J. S., and Kopan, R. (2000) *Dev. Biol.* 223, 151–153
- Deleted in proof
- Deleted in proof
- Capell, A., Steiner, H., Rottig, H., Keck, S., Baader, M., Grim, M. G., Baumeister, R., and Haass, C. (2000) *Nat. Cell Biol.* 2, 205–211
- Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) *Nature* 398, 513–517
- Armstrong, M., Petit, A., Vincent, B., Scarsella, S., Alves Da Costa, C., and Chelier, F. (2001) *Nat. Cell Biol.* 3, 1080–1083
- Berzovska, O., Jadt, C., McLean, P., Aster, J. C., Hieck, C., Xia, W., Wolfe, M. S., Kimberly, W. T., Wilmaster, G., Selkoe, D. J., and Hyman, B. T. (2000) *J. Neurochem.* 76, 583–583

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